The habenula clock influences response to a stressor

Adriana Basnakova\textsuperscript{a,b}, Ruey-Kuang Cheng\textsuperscript{c}, Joanne Shu Ming Chia\textsuperscript{e}, Giuseppe D’Agostino\textsuperscript{c}, Suryadi\textsuperscript{d}, Germaine Jia Hui Tan\textsuperscript{e}, Sarah R. Langley\textsuperscript{c}, Suresh Jesuthasan\textsuperscript{b,c,}\textsuperscript{*}

\textsuperscript{a} School of Biological Sciences, University of Manchester, UK
\textsuperscript{b} Institute of Molecular and Cell Biology, Singapore
\textsuperscript{c} Lee Kong Chian School of Medicine, Nanyang Technological University, 59 Nanyang Drive, 639721, Singapore
\textsuperscript{d} School of Physical and Mathematical Sciences, Nanyang Technological University, Singapore

\textbf{ARTICLE INFO}

Keywords:
Anxiety
Circadian clock
Habenula
Spontaneous activity
Predictive coding

\textbf{ABSTRACT}

The response of an animal to a sensory stimulus depends on the nature of the stimulus and on expectations, which are mediated by spontaneous activity. Here, we ask how circadian variation in the expectation of danger, and thus the response to a potential threat, is controlled. We focus on the habenula, a mediator of threat response that functions by regulating neuromodulator release, and use zebrafish as the experimental system. Single cell transcriptomics indicates that multiple clock genes are expressed throughout the habenula, while quantitative in situ hybridization confirms that the clock oscillates. Two-photon calcium imaging indicates a circadian change in spontaneous activity of habenula neurons. To assess the role of this clock, a truncated clock gene was specifically expressed in the habenula. This partially inhibited the clock, as shown by changes in per\textsuperscript{2} expression as well as altered day-night variation in dopamine, serotonin and acetylcholine levels. Behaviourally, anxiety-like responses evoked by an alarm pheromone were reduced. Circadian effects of the pheromone were disrupted, such that responses in the day resembled those at night. Behaviours that are regulated by the pineal clock and not triggered by stressors were unaffected. We suggest that the habenula clock regulates the expectation of danger, thus providing one mechanism for circadian change in the response to a stressor.

1. Introduction

Expectations play a major role in determining the response of a person or animal to a sensory stimulus or situation. In this framework, known as predictive coding or active inference, the brain is not a passive recipient of stimuli, but actively generates expectations that are updated by experience (Barrett, 2017; Friston, 2018). Thus, encountering a cue such as the smell or sound of a predator increases the expectation of danger, turning a normally innocuous stimulus into a threat. Abnormal expectations, or predictions, have been proposed to underlie stress-related conditions such as depression (Smith et al., 2021) and post-traumatic stress disorder (Linson and Friston, 2019; Wilkinson et al., 2017). Predictions are mediated in part by spontaneous activity in the brain (Berkes et al., 2011; Koren and Denève, 2017; Pezzulo et al., 2021), while updating of expectations is enabled by the release of neuromodulators. Dopamine and serotonin contribute to the comparison of current input with pre-existing expectations. When outcomes surpass predictions, dopamine release by neurons in the midbrain increases; when there is disappointment, dopamine release is inhibited (Schultz, 1998). Serotonin release by the raphe provides an indication of the magnitude of the prediction error (Matias et al., 2017). Acetylcholine appears to have a role in setting the precision of predictions (Moran et al., 2013). Behaviour and emotional states can thus be understood to be the result of genetically encoded programs that are continuously modified by predictions, enabling the animal to respond optimally to perceived reality.

The habenula, which contains several subdomains, has emerged in recent years as an important regulator of neuromodulators involved in predictive coding. The medial habenula in zebrafish (Hong et al., 2013), like that of mouse (Ren et al., 2011), includes cholinergic neurons. Disruption of specific subdomains of the medial habenula leads to anxiety-like behaviour that is experience dependent (Agotsuma et al., 2010; Lee et al., 2010). The lateral habenula regulates midbrain dopaminergic and serotoninergic neurons, and phasic activity occurs here when there is an unexpected punishment or absence of reward (Matsumoto and Hikosaka, 2009). As an animal learns the actions that...
2. Materials and methods

2.1. Single cell data reanalysis

Count tables were downloaded from Gene Expression Omnibus, accession number GSE105115. All processing downstream was done using R/BioConductor (R 4.0.2/Bioconductor 3.12) packages designed to handle single cell data using the SingleCellExperiment class (v. 1.12.0) (Amezquita et al., 2020). A detailed explanation with code and figures is provided as a GitHub repository at https://github.com/langlalora/habenula_reanalysis. Briefly, gene-level count tables from the SmartSeq2 experiment were merged retaining the information on the plate of origin. Genes that were not detected in any barcode were removed. Barcodes were discarded according to the following criteria: low expression, low number of genes detected, high percentage of mitochondrial expression and high percentage of ribosomal transcript expression. Thresholds for every criteria were set as 3 Mean Absolute Deviations (MADs). Outlier calculations were done separately in each plate using the \texttt{getTopHVGs} function from \texttt{scran}. These were used as an input to perform dimensionality reduction via Principal Component Analysis as implemented in the \texttt{BiocSingular} package (v. 1.4.0, (Lun, 2020b)).

For the 10X datasets (larval) no filtering was performed as the distribution of QC metrics was well within standard ranges, suggesting data were already filtered. Normalization, HVG estimation and dimensionality reduction were performed in the same way as described above, with the exception that there was no batch effect to account for and correct. An additional doublet detection step was included using the \texttt{scDblFinder} package (v. 1.4.0, (Germain et al., 2020)), which flagged 172
barcodes as doublets and 4193 as singlets. Doublets were not removed at this stage of the analysis. The intrinsic dimension estimation (k = 66) yielded 12 dimensions to perform SNN graph clustering, which was performed using 5, 10, 15, 20, and 30 neighbours. In parallel, 36 dimensions were also considered as done by the authors of the original publication. For the 12-dimensional space, 5 and 10 neighbours were considered as they yielded numbers of clusters similar to those reported in the publication; for the 36-dimensional space, 10 and 15 neighbours were considered. However, in both 12 and 36 dimensions a cluster was entirely made up of doublets, so doublets were removed and the whole process (normalization, HVG selection and dimensionality reduction) was repeated. Cluster separation metrics (approximate Silhouette and modularity) favoured clustering with 10 neighbours (12 dimension) and 15 neighbours (36 dimensions). To reproduce the original clustering results the AUCell package was used, and Jaccard indices were used to match clusters to labels. In order to improve the label assignment, cells whose top AUC label showed less than a 20% difference from the second best label were classified as “ambiguous”. Ambiguous labels were then reassigned using the closest centroid in the approximate silhouette width calculation, thus making the labels conform more to the transcriptional space. Improvements in the reassignment of these labels were measured by comparing silhouette widths and the distribution maximum Jaccard indices for every cluster-AUC label pair before and after reassignment. Both metrics showed an improvement in both silhouette width and median best Jaccard index upon reassignment.

2.2. Transgenic lines

TgBAC(gng8:GAL4)-D26 (Hong et al., 2013) or Tg(1:1011c) (Scott and Baier, 2009) was used to drive expression in the habenula. Calcium imaging was performed using Tg(elav3:GCaMP6f) and Tg(UAS:GCaMP6f) (Scott and Baier, 2009). Tg(UAS:EGFP-2A-∆clocka-5×MYC) (Abbreviated here as Tg(UAS:EGFP-2A-∆clocka) was generated by injection of pT2-UAS:EGFP-2A-∆clocka-5×MYC using Tol2 mediated transgenesis. All experiments were carried out under guidelines approved by the IACUC of Biopolis (181408) and NTU (A19014). The minimal number of animals was used. Fish were maintained in a Techniplast system, with a light/dark cycle of 14/10 h and a water temperature of 28 °C.

2.3. Two-photon imaging

2.3.1. Extended imaging

Based on the methods described by (Leung et al., 2019), non-anesthetized and non-paralyzed larvae were mounted in 2% low melting agarose (LMA), in a glass-bottom dish (Matek). The dish was filled with E3 medium supplemented with 1% Hepes and bubbled continuously with carboxygen (95% oxygen). Imaging was performed with a Nikon A1R MP system, attached to an upright FN1 microscope. Imaging was performed using 2×1.0 NA water dipping objective. Resonant scanning was performed with 2× averaging, and 20 focal planes were collected, spaced 5 μm apart. Signal was detected with a GaAsP detector. A piezo drive (Mad City Labs) was used for fast focusing during imaging. 21 stacks were collected, at an interval of 1 s, followed by a delay of 1 h. The maximum imaging duration was 29 h. After imaging, larvae were kept in the dark. They were then released from the agarose. Only data from fish with normal blood flow and active swimming upon release were used for analysis.

To obtain a measure of calcium levels in the whole habenula, fluorescence intensities were summed at each time point using Fiji. The intensity at each hour was averaged and z-scores were plotted as a function of zeitgeber time. Statistical analysis was performed with Prism, by nonlinear regression to a sine wave.

2.3.2. Short duration imaging

6-day-old Tg(elav3:Z2B-GCaMP6f) or Tg(1:shp70[Gal4-VI]) (Chan et al., 2011), Tg(UAS:GCaMP6f) larvae were immobilized with mivacurium, mounted in 2% low melting temperature agarose and imaged with the two-photon system described above, using resonant scanning at a single plane. Frame size was 512 x 256 pixels and 2× averaging was used, leading to a frame rate of 14.5 Hz. Segmentation of cells expressing cytoplasmic GaMP6f was performed manually in Fiji, while segmentation of cells expressing nuclear GaMP6f was performed automatically with Suite2p (Stringer and Pachitariu, 2019). Z-scores were obtained for each cell in a recording, and cells were clustered using K-means clustering in Python. The classification was performed using maximum likelihood and leave-one-out cross-validation. Specifically, at each iteration we removed one dataset from the group and constructed empirical cluster distributions for both categories (ZT3 and ZT15) from the remaining datasets. The removed dataset was then assigned to the category whose empirical distribution maximized its likelihood. This process was repeated independently until all datasets were classified.

2.4. Immunohistochemistry and imaging of EGFP-∆CLK

The Tg(gng8:GAL4, UAS:EGFP-Δclocka) larvae at the age of 7 dpf were fixed in 4% PFA at 4 °C for ~22 h. For immunostaining of extracted brains, skin covering the brain was manually removed and exposed brains were washed 3 times in PBST. Antigen retrieval was performed by incubating the fish in 150 mM Tris-HCl (pH 9.0) for 5 min at room temperature in order to equilibrate, and by subsequent heating of the samples at 70 °C for 15 min. Following several washes and a blocking step, samples were incubated in the rabbit anti-GFP primary antibody (Torrey Pines Biolabs Inc., #TP401), diluted 1:500, and the mouse anti-Myc antibody (9E10; Santa Cruz Biotechnology), diluted 1:100, at 4 °C for a few days. The secondary antibodies used were Alexa Fluor 488 goat anti-rabbit (ThermoFisher and Abcam), diluted 1:1000, and Alexa Fluor 647 goat anti-mouse (ThermoFisher), diluted 1:500. The immunostained brains were mounted in 2% LMA in PBS and imaged using a Zeiss LSM800 confocal microscope equipped with 40X 0.7 NA water immersion objective.

2.5. Screening and selection of Tg(gng8:GAL4, UAS:EGFP-Δclocka) fish

All of the Tg(gng8:GAL4, UAS:EGFP-Δclocka) fish used in the following experiments were first screened for GFP fluorescence at 3 dpf using an Olympus MVX10 or Zeiss V16 stereo fluorescence microscope. Since the expression of EGFP-ΔCLK in the habenula was mosaic, only individuals with expression in the majority of habenula cells were selected and used for further experiments as ΔCLK-positive fish. To maximize expression, fish were generated by in-crossing. A consequence of this, however, is that fish with zero expression were rare. Thus, in addition to individuals with zero expression, larvae exhibiting a minimal number of randomly labelled cells (less than 15 cells per fish) were used as the control siblings (Fig. S1). Despite this, the size of the control group was smaller than the ΔCLK group.

Following the behavioural experiments, larvae were immobilized in tricaine methanesulfonate (MS-222; final concentration of 0.01 mg/mL; Sigma), mounted in 2% LMA in E3 medium and the habenula-specific expression of EGFP was assessed using a Zeiss LSM800 confocal microscope and 40X water dipping objective.

2.6. Quantification of DA and 5-HT levels

Three-month old female ΔCLK fish and their control siblings were immobilized usingiced water and euthanized by decapitation at ZT3 and
2.7. Behaviour assays

2.7.1. Acoustic startle following exposure to Schreckstoff

Thirty-six to sixteen-day-old ΔCLK larvae and their non-expressing siblings were placed in two transparent plastic tanks with dimensions of 80 × 54 x 33 (L × W × H in mm) and their locomotor activity was recorded from above at 10 fps using a camera (acA2040-90μm USB 3.0; Basler). Each of the two tanks was filled with 20 mL of facility water and held 6–8 fish. The water level in each tank was 5 mm in height to minimize vertical movement. The alarm substance was freshly prepared from adult zebrafish each week (Matharu et al., 2012), and was tested for effectiveness on adults before use on larvae. An Arduino Grove Vibration Motor was positioned in between the two plastic tanks, on the stage holding the tanks, to provide the stimulus. The vibration was 1 s in duration and was presented 3 times, at 1 min intervals, starting 5 min before and after the delivery of the Schreckstoff. A startle response was defined as a change in body orientation of more than 90° within 5 s in response to each vibration tone. The response (yes or no for each fish) was averaged across 3 tones for each group to obtain a response percentage before and after the delivery of the Schreckstoff. Both the vibration tones and the delivery of Schreckstoff were controlled by an Arduino board, connected with the video-recording Python codes. Experiments were performed on 3 separate batches of larvae. A different batch of freshly prepared skin extract was used for each experiment; the extract was verified for effectiveness by testing on adult fish. Only extract that evoked freezing was used.

2.7.2. Locomotor activity and sleep analysis

The Tg(tgmy8:GAL4,UAS:EGFP-Δclocka) larvae were raised under standard conditions. Each experimental run involved animals from the same batch, originating from one pair of parents. The larvae were screened and selected as described in the previous section. At the age of 6 dpf the larvae were placed individually in a 24-well plate filled with 1.5 mL of tank water per well. The plate was then transferred into an incubator equipped with an IR camera (acA2040-90μm USB 3.0: Basler), a white LED box and four IR LED bars (LBS2-00-080-3-IR850-24V, 850 nm: TMS Lite). The camera and the LEDs were connected to a PC via a microcontroller board (Arduino Uno NO). At the onset of recording (ZT6 – 9), fish were kept under ambient light, which was switched off at ZT14 and was kept off during the next day for the monitoring of activity under DD. A customized script written in Python 2.7 (see https://github.com/rkcheng/SleepRecording-and-CircadianStartleResponse), incorporating functions from the OpenCV library was used to control the Arduino microcontroller and simultaneously video-track fish locomotor activity. The video was captured using 576 × 864-pixel resolution at 16 frames per second and the background subtraction method was implemented to extract the ‘x’ and ‘y’ coordinates of each fish online. The position data was then converted to total distance (mm) travelled by each fish per 1 min time. A sleep bout (or a rest bout in the subjective day or light phase) is defined as a continuous period of inactivity lasting 1 min or longer. The threshold of detecting any inactive minute is set to no more than 1 s active in a given minute. The data conversion, further analysis and plotting were carried out using custom-written Excel macros, Python 2.7 and Estimation Statistics (Ho et al., 2019).

2.7.3. Arousal threshold

Six days-old ΔCLK larvae and their control siblings were randomly placed in a 24-well plate and their locomotor activity was video-tracked as described in the previous section. In order to assess the arousal during sleep, vibration stimuli were applied through a speaker controlled, via an Arduino microcontroller board, by a modified version of the activity-tracking code in Python 2.7. Starting at ZT17, eighteen stimuli of nine different intensities, where 50% of computer audio output represented the highest intensity, were delivered for 200 ms (starting from 0%) in 12–16% increments every minute, first in a descending and then ascending order. The time interval between stimuli was determined based on previous findings that suggest a 30-s inter-stimulus gap is sufficient to avoid behavioural habituation (Burgess and Granato, 2007). The protocol was repeated at the commencement of every hour until ZT22, while the onset by ascending or descending order was altered each time. In total, 12 replicates (i.e. technical replicates) of each stimulus intensity were generated. A fish was scored as responding if it moved more than 7 pixels (~4 mm) following the stimulus delivery and the average percentage of responding fish was calculated for each of the stimulus intensities (Woods et al., 2014). The observed values were corrected for variations in the baseline locomotor activity, by first calculating the background probability of locomotion as a percentage of larvae which moved more than 7 pixels 5 s before the application of each stimulus. The average of 108 measures of the background probability of locomotion was then used to calculate the corrected probability of response at each of the stimulus intensities, implementing the following equation: corrected probability of response = observed value x (observed value - background offset)/(max observed value - background offset).

2.7.4. Novel tank assay

All experiments were conducted between ZT2 and ZT4 on 3–5 month-old adult zebrafish, as described elsewhere (Haghani et al., 2019). Fish were recorded in pairs in a darkened room with a black background, where tanks were illuminated from the top with a natural light LED bar. Novel tanks were made of glass measuring 20 cm (L) by 5 cm (W) by 12 cm (H) with opaque sides (to prevent fish from viewing each other), placed side by side. Fresh water from the facility was added to tanks up to the 10 mL mark to make a total volume of 1 L. The setup was placed within a curtained area to obscure the experimenter.

Prior to exposure to the novel tank, fish were handled carefully to ensure that handling stress was minimized. Fish were gently netted in pairs from their home tanks in the facility to smaller crossing tanks, where they were brought into the room with the behavioural setup. Then, fish were netted into 100 mL beakers with ~30 mL of fish facility water and quickly poured into the novel tank. The recording was started within 30 s of fish being in the recording tanks. For exposure to Schreckstoff, fish were placed in 100 mL beakers containing 200 μL of heated skin extract (Chia et al., 2019) in 50 mL fish facility water for 2 min. Fish were netted into another beaker containing fresh facility water before being poured into the novel tank. Videos were acquired at 10 frames per second for 600 s (10 min) using a Basler Ace camera (acA1300–200 μm; 1280 × 1024). The position (x-y coordinates) of the fish were tracked, and speed was binned by mm/s into a probability density curve.

For acute exposure to Schreckstoff, fish were first habituated in the glass tanks for 5–7 min. Then, fish were recorded in the tanks for 5 min before 1 mL of 1:4 dilution of heated skin extract was pipetted into the tanks. The recording continued for 5 min after Schreckstoff delivery for the alarm response to be observed. Two fish were tested at one time, each in a separate tank, with one tank containing ΔCLK fish and the other a non-expressing sibling. The tank used for a particular genotype was switched at every trial. Characteristic alarm responses were quantified, such as time spent in the bottom third of the tank, number of darting episodes (fish speed >5 SD above average swim speed for the first 5 min), and number of freezing episodes (movement of <5.5 mm
per second). Tracking was automated to prevent observer bias. Sample size was based on previous experiments (Chia et al., 2019).

2.7.5. Light/dark choice assay

All experiments were performed between ZT2 and ZT9 in a behavioural arena within blackout curtains as described previously (Cheng et al., 2016). Four transparent plastic tanks (dimensions: 43 mm W × 60 mm L × 30 mm H) filled with 30 mL fresh tank water served as assay chambers. Opaque cardboard sheets were placed between the 4 tanks. Tanks were placed on an Apple iPad screen and videos (9 fps) were recorded on a USB3.0 Basler camera (Model# acA2040-90umNIR 1440 × 1080 pixels) with a long-pass filter (MIDOPT, LP830 830 nm) placed above. Two IR light bars (850 nm peak from TMS-lite) positioned next to the 4 tanks served as an IR light source. The light/dark compartments were created by white and black rectangles (with 50% transparency level for the black) on a Microsoft PowerPoint slide displayed on the iPad Air at highest brightness level. Sample size was based on previous experiments (Cheng et al., 2016).

Larvae were exposed either to Schreckstoff or to control water (embryo medium) in a treatment tank of the same dimensions with the same volume of tank water for 10 min. Larvae were then gently pipetted from the treatment tank into two wash-out tanks sequentially before being placed into the dark/light test chamber. Real-time tracking of the fish was achieved by custom-written Python codes using the OpenCV library and recorded as coordinates of the centroid of the fish across time. Larval behaviour was recorded for 10 min. Offline analysis was performed using custom-written Excel macros to determine the position of the animals, the number of entries into each compartment, the percentage of total time spent in each compartment, etc. A few fish (N = 2 in both the Control group and Gng8/ΔCLK group) were excluded from the analysis because they were entirely immobile or because of tracking errors.

2.8. In situ hybridization

Hybridisation chain reaction with split initiator probes was conducted using the HCR v3.0 protocol for whole-mount zebrafish embryos and larvae from Molecular Instruments. Probe hybridization buffer, amplification buffer, probe wash buffer, probe sets and HCR amplifiers and larvae from Molecular Instruments. Probe hybridization buffer, conducted using the HCR v3.0 protocol for whole-mount zebrafish embryos formed using custom-written Excel macros to determine the position of the larvae within the assay chamber. Opaque cardboard sheets were placed between the 4 tanks. Tanks were placed on an Apple iPad screen and videos (9 fps) were recorded on a USB3.0 Basler camera (Model# acA2040-90umNIR 1440 × 1080 pixels) with a long-pass filter (MIDOPT, LP830 830 nm) placed above. Two IR light bars (850 nm peak from TMS-lite) positioned next to the 4 tanks served as an IR light source. The light/dark compartments were created by white and black rectangles (with 50% transparency level for the black) on a Microsoft PowerPoint slide displayed on the iPad Air at highest brightness level. Sample size was based on previous experiments (Cheng et al., 2016).

Larvae were exposed either to Schreckstoff or to control water (embryo medium) in a treatment tank of the same dimensions with the same volume of tank water for 10 min. Larvae were then gently pipetted from the treatment tank into two wash-out tanks sequentially before being placed into the dark/light test chamber. Real-time tracking of the fish was achieved by custom-written Python codes using the OpenCV library and recorded as coordinates of the centroid of the fish across time. Larval behaviour was recorded for 10 min. Offline analysis was performed using custom-written Excel macros to determine the position of the animals, the number of entries into each compartment, the percentage of total time spent in each compartment, etc. A few fish (N = 2 in both the Control group and Gng8/ΔCLK group) were excluded from the analysis because they were entirely immobile or because of tracking errors.

Larvae were exposed to Schreckstoff either to Schreckstoff or to control water (embryo medium) in a treatment tank of the same dimensions with the same volume of tank water for 10 min. Larvae were then gently pipetted from the treatment tank into two wash-out tanks sequentially before being placed into the dark/light test chamber. Real-time tracking of the fish was achieved by custom-written Python codes using the OpenCV library and recorded as coordinates of the centroid of the fish across time. Larval behaviour was recorded for 10 min. Offline analysis was performed using custom-written Excel macros to determine the position of the animals, the number of entries into each compartment, the percentage of total time spent in each compartment, etc. A few fish (N = 2 in both the Control group and Gng8/ΔCLK group) were excluded from the analysis because they were entirely immobile or because of tracking errors.

2.8.1. qHCR imaging

4 larvae from each sample were mounted in 2% LMA in PBS in dorsal view and imaged using a Zeiss LSM800 upright confocal microscope with 40× water dipping objective at 0.5× zoom. The acquisition settings were identical in all experiments. Z-stacks were collected at 512 × 512 resolution, giving a x-y pixel size of 0.62 μm and a z-step of 0.73 μm.

Sample size was based on Trivedi et al., 2018.

2.8.2. Image analysis

To quantify gene expression in the entire habenula across different fish, segmentation of habenula was performed manually using the Segmentation Editor in Fiji (Schindelin et al., 2012). The integrated intensity of per3 and arntl1b was measured using the 3D Objects Counter (Bolte and Cordelières, 2006). The ratio was calculated to compensate for potential tube-tube variation as well as change in intensity with imaging depth. Values were plotted using GraphPad Prism.

To compare the level of per3 in ΔCLK expressing and non-expressing cells, within-fish analysis was performed on single planes. ΔCLK cells were segmented by thresholding the GFP signal, while the habenula was manually segmented. Masks for control cells were obtained by subtracting the ΔCLK mask from the habenula mask. ROIs were then created, and mean intensity measured, based on methods described by Choi et al., 2018, i.e. with a mean filter of 3 and background correction. Statistical analysis was performed with estimation statistics (Ho et al., 2019).

2.8.3. dHCR imaging

Following label for cry1a, 3 larvae from each of 6 time-points were imaged with confocal microscopy, using an Achroplan 63× water dipping objective and 2048 × 4093 image size, giving a x-y pixel size of 0.0495 μm z step was 0.78 μm. Sample size was based on Choi et al., 2018. To analyse the images, the habenula in one plane ~10 μm from the surface was manually segmented. Spots were identified in Fiji using a Laplacian transform with FeatureJ, with the smoothing scale set at 5. The acquisition settings were identical for acquisition and analysis of all dHCR images.

3. Results

3.1. Multiple clock genes are expressed in the zebrafish habenula

Using in situ hybridization in brain sections with DIG-labelled probes, a number of clock genes, such as per1b and bmal (arntl1a), have been shown to be expressed in the habenula of adult zebrafish (Weger et al., 2013). To better characterize clock gene expression in this structure, we analysed single cell transcriptomes of the zebrafish habenula (Pandey et al., 2018). A diversity of clock gene components, including arntl1b, arntl2, per1a, per2, per3, cry1a, cry1ab, cry1b, clocka, clockb and nr1d1 were found (Fig. 1). These transcripts were
isolated from gng8-expressing habenula neurons, which are found throughout the habenula (Hong et al., 2013; Pandey et al., 2018). All clusters contained clock genes, suggesting that the molecular clock is broadly distributed.

To examine the temporal dynamics of the clock in larval zebrafish, quantitative in situ hybridization using hybridization chain reaction (HCR) (Choi et al., 2018) was carried out on fish that were fixed at 6 different time points (Fig. 2). With qHCR imaging, arntl1b appeared to be expressed at a higher level in the habenula compared to the rest of the brain (Fig. 2A), with a stronger signal at approximately zeitgeber time ZT 15; expression at ZT 19 and ZT 23 was detected at the posterior margins. per3 was detectable broadly in the brain, including the entire habenula, with strongest expression at ZT 23 and ZT 3 (Fig. 2B). Signal intensity obtained with qHCR imaging provides a relative measure of transcript level (Choi et al., 2018), and can vary with sample thickness and between tubes. To minimize error from these potential confounds, we calculated the ratio of arntl1b/per3 as a means of comparing gene expression between animals. This ratio, which also maximizes sensitivity given the antiphasic expression of the genes, showed a circadian variation with a peak at ZT 11–15 (Fig. 2D). To test whether clock gene cycling requires light, we examined expression in fish grown in constant darkness. The ratio of arntl1b/per3 expression was maximal at CT 15 and minimal at CT 3 (Fig. 2E–G). We used single molecule imaging, with dHCR imaging of cry1a, to further test whether the clock is cycling. Again, a circadian change in expression level was detected in the habenula in constant darkness (Fig. 2H and I), with a peak at CT 3 and a trough at CT 15, similar to what has been reported in other tissues under LD (Cavallari et al., 2011) or DD conditions (Kobayashi et al., 2000). These observations confirm previous findings using clock reporters (Wang et al., 2020; Weger et al., 2013) that the molecular clock is active in the zebrafish habenula.

3.2. The zebrafish habenula displays circadian variation in intracellular calcium

The presence of the molecular clock should lead to a number of circadian changes in a cell. One such change is the level of cytoplasmic calcium (Pennartz et al., 2002). To examine intracellular calcium levels
in the zebrafish habenula, two-photon imaging was carried out in fish held in constant darkness. Confocal microscopy was not used to avoid light-induced disruption of the molecular clock by visible light (Tamai et al., 2007). By imaging a series of z-stacks every hour, such that the entire habenula could be captured (Fig. 3A–D), we observed a decrease in fluorescence intensity during the subjective night and an increase during the subjective day (Fig. 3E; 5 out of 6 fish imaged). The average trace fitted a sine wave (brown line in Fig. 3E) with a period of 20.5 h [95% CI 19.7, 21.5; R² = 0.869] and baseline of 0.0 [95% CI -0.17, 0.07]. This finding is consistent with the hypothesis that the habenula contains an active molecular clock that has the potential to drive changes in the intracellular calcium even in the absence of light, a prominent circadian zeitgeber.

In the mouse, neurons in both medial (Sakhi et al., 2014a) and lateral
3.3. Expression of a truncated clock gene in the habenula affects function

The expression of clock genes and the circadian change in intracellular calcium in the habenula raises the possibility that an intrinsic clock influences habenula function. To test this, we selectively expressed a truncated clocka gene (Dekens and Whitmore, 2008), referred to as Δclocka, in the habenula. This construct has been shown to act in a dominant manner to inhibit the molecular clock in the zebrafish pineal (Livne et al., 2016). Δclocka was expressed in the habenula using the GALA/UAS system with TgBAC(mug8:GAL4) (Hong et al., 2013) as the driver. In addition to Δclocka, the effector construct also included enhanced green fluorescent protein (EGFP), which was separated from ΔClocka (ΔCLK) by a 2A peptide linker, thus enabling the expression of two separate proteins. The presence of EGFP in the habenula neurons of Tg(mug8:GAL4, UAS:EGFP-Δclocka) fish (Fig. 4A and B) indicates that the construct is expressed appropriately and enables convenient identification of ΔCLK-positive individuals (Livne et al., 2016). Labelling with an antibody against the Myc tag, which is fused in frame at the C-terminus of ΔCLK, indicates that the truncated protein is translated (Fig. 4C). Cells expressing ΔCLK would be expected to have a lower level of Clock-regulated genes such as per, than non-expressing cells. To assess this, qHCR imaging was performed on larvae with scattered expression of ΔCLK and the level of per3 in cells with EGFP expression was compared with the ratio in non-expressing cells in the same sample. As seen in Fig. 4D the level of per3 differed between ΔCLK and control cells at all time points, with a paired Cohen’s D of 1.58 [95.0%CI 1.03, 4.22; p = 0.0049 paired t-test] at CT3, 0.525 [95.0%CI 0.337, 4.25; p = 0.0115] at CT7, -1.89 [95.0%CI -4.78, -0.734; p = 0.0054] at CT11, -1.57 [95.0%CI -3.38, -1.15; p = 0.0185] at CT15, 0.872 [95.0%CI 0.636, 2.26; p = 0.0017] at CT19 and 0.872 [95.0%CI 0.536, 0.816; p = 0.0167] at CT23. This suggests that expression of ΔCLK influences the molecular clock, although it does not eliminate cycling.

Given the partial inhibition shown by gene expression analysis, we asked whether there is any evidence that expression of ΔCLK affects habenula function. The habenula regulates the broad release of serotonin and dopamine (Amo et al., 2010; Satar et al., 2020) and contains...
from extracts were analysed by liquid chromatography and mass spectrometry (LC/MS) (Chatterjee and Gerlai, 2009). While non-expressing fish had a clear difference in the day- and night-time levels of dopamine (Fig. 4E; mean difference = 124 pg/mg, p < 0.008 by Students t-test), there was almost no difference in fish expressing ΔCLK protein (mean difference = 6.3 pg/mg, p = 0.547 by Student’s t-test). The mean difference in between day and night-time levels of serotonin and acetylcholine levels was also higher in control fish (117 pg/mg and 454 pg/mg respectively, p = 0.002 and 0.001 by Student’s t-test), compared to ΔCLK-positive fish (68.1 pg/mg and 137 pg/mg respectively, p = 0.001 by Student’s t-test in both cases). This change of neuromodulator levels is consistent with the notion that expression of ΔCLK in the habenula affects function.

3.4. Habenula expression of ΔCLK reduces anxiety-like behaviour induced by the alarm substance

We hypothesized that a stress response would be affected by the expression of ΔCLK in the habenula. One such response for fish is the alarm response, which is triggered by the pheromone Schreckstoff. In adult zebrafish, Schreckstoff causes an acute change in swimming behaviour (Jesuthasan and Matharu, 2008; Speedie and Gerlai, 2008). Following transient exposure to Schreckstoff, fish display abnormal scototaxis (Maximinio et al., 2014) and heightened vigilance. To determine whether manipulation of the habenula clock influenced the acute response, adult fish were placed individually in a test tank. After a period of acclimatization, the alarm substance was introduced into the tank (Fig. 5A). Adult Tg(gng8:GAL4, UAS:EGFP-Δclocka) zebrafish showed an increase in darting (Fig. 5C; paired mean difference of 1.33 s [95.0% CI 0.5, 2.67], p = 0.0260 Wilcoxon rank sum test), as did their non-expressing siblings (paired mean difference of 1.25 s [95.0% CI 0.417, 3.33], p = 0.0256 Wilcoxon rank sum test). There was no difference in the movement to the tank base (Fig. 5D; paired mean difference of 16.4 [95.0% CI 1.02, 41.2], p = 0.530 for ΔCLK fish and 12.6 [95.0% CI -0.997, 28.1], p = 0.182 for siblings).

To monitor expectation of danger (or anxiety-like behaviour), individual fish were exposed to the alarm substance in a beaker, then transferred to a novel tank containing fresh water (Fig. 5E). Control fish showed a reduction in speed (Fig. 5F), which is stronger in the first 2 min after transfer (Fig. 5G; Cohen’s d of 1.69 [95%CI 0.81, 2.52], p = 0.00538 Mann Whitney). We also monitored the post-exposure response of larval zebrafish, using a light-dark choice assay as an indicator of stress (Steenbergen et al., 2011) (Fig. 5H). Larval Tg(gng8:GAL4, UAS:EGFP-Δclocka) fish displayed less dark avoidance after transient exposure to Schreckstoff, as indicated by the increased amount of time spent in the dark side of the tank (Fig. 5I and J; mean difference of 16.4 [95.0% CI 1.02, 41.2], p = 0.0256 Wilcoxon rank sum test). There was no difference in the movement to the tank base (Fig. 5D; paired mean difference of 16.4 [95.0% CI 1.02, 41.2], p = 0.530 for ΔCLK fish and 12.6 [95.0% CI -0.997, 28.1], p = 0.182 for siblings).

We next asked whether the expectation of danger induced by the alarm substance varies in a circadian fashion in zebrafish; a circadian expression of clocka affects function. (Hong et al., 2013). If expression of ΔCLK has any effect on habenula function, the global levels of secreted serotonin, acetylcholine and dopamine, which vary in a circadian fashion (Hut and Van der Zee, 2011; Mendoza, 2017), should be affected. To test this, brains were isolated from adult zebrafish expressing EGFP-ΔCLK and from their non-expressing siblings, at ZT3 and ZT15. The supernatant

Fig. 4. The effects of expressing truncated clocka in the habenula. (A) Specific expression of EGFP in the habenula of a Tg(gng8:GAL4, UAS:EGFP-Δclocka) fish. (B) At higher magnification, EGFP is visible in the cytoplasm of habenula neurons. (C) Detection of Myc, which is fused to the C-terminus of ΔCLK, in the habenula of transgenic fish. (D) Level of per3 in the habenula of fish with mosaic expression of EGFP-ΔCLK, as determined by qHCR imaging. The lower graph shows Cohen’s d, indicating the degree of difference between ΔCLK-expressing and non-expressing cells at each time point. Values are given in the main text. (E) Effects of ΔCLK expression in the habenula on global neuromodulator levels. Plot showing the amount of secreted serotonin, dopamine and acetylcholine in the brain of and Tg(gng8:GAL4, UAS:EGFP-Δclocka) and sibling fish (N = 3) at ZT3 and ZT15. Fish with ΔCLK expression in the habenula have a reduced day-night change in levels. Scale bar = 100 μm in panel A, 20 μm in panels B and C. * indicates p < 0.05; ** indicates p < 0.005 by paired t-test. Exact p values are provided in the text.
Fig. 5. The effects of the alarm substance Schreckstoff on Tg(gng8:GAL4, UAS:EGFP-Δclocka) fish. (A-D) Acute effects in adult fish (N = 12). (A) Schematic diagram of the experiment. (B) Speed distribution in fish before and after exposure to the alarm substance, where the substance remained in the tank after delivery. (C-D) Time spent darting (C), restricted to the tank base (D) in the presence of the alarm substance. As indicated by the paired mean difference plots (lower plot), fish with and without expression of truncated clocka behave similarly. (E-G) Post-exposure behaviour in adult fish (N = 8). (E) Schematic diagram of the experiment. (F) Speed distribution in a novel tank containing clean water, after exposure to alarm substance or clean water. (G) Distance traversed in a novel tank containing clean water, at different times after exposure to the alarm substance. (H-J) Post-exposure behaviour in 2 week-old zebrafish. (H) Schematic diagram of the experiment. (I) Percentage of time in the dark area of a tank, after 10 min of exposure to embryo water. (J) Percentage of time spent in the dark side after exposure to the alarm substance. Tg(gng8:GAL4, UAS:EGFP-Δclocka) fish spend more time in the dark side, with a Cohen’s D of 1.2.
freezing evoked by the alarm response is circadian in zebrafish and is influenced by the habenula clock.

3.5. Expression of ΔCLK in the habenula does not affect pineal-regulated circadian behaviour

We hypothesized that while the circadian clock in the habenula affects the response to a stimulus that influences expectation, circadian behaviours that are under the control of the pineal clock (Livne et al., 2016) should be unaffected. To test this, we analysed the rhythmic locomotor activity of ΔCLK-positive larvae and their non-expressing siblings. In a normal LD cycle, movement of ΔCLK larvae and siblings were similar (Fig. S2). We next monitored the activity of larvae in constant darkness, to eliminate the masking effects of light and dark on clock-regulated behaviour (Livne et al., 2016). All larvae were entrained to the standard LD cycle before being monitored under constant darkness (DD). As seen in Fig. 7A–E and Table S1, ΔCLK larvae showed a similar pattern of locomotion as their non-expressing siblings, becoming mobile during the light phase and during the subjective day, while exhibiting quiescent periods of inactivity during the 1st and 2nd subjective night of the recording. The amount of activity during the subjective day was similar between ΔCLK and control fish, as measured by sleep bout duration (Fig. S3A; p = 0.99 for first subjective day and p = 0.51 for second subjective day, by Welch’s t-test) and amount of rest (Figs. S3B–D; Table S2). During the subjective night, the amount of sleep was similar between ΔCLK and control fish, as measured by sleep bout duration (Fig. 7E; by Welch’s t-test, p = 0.54 by for first night, p = 0.32 for second night), frequency of sleep (Fig. 7F; p = 0.61 for first night, p = 0.53 for second night) and total sleep (Fig. 7G; p = 0.69 for first night, p = 0.24 for second night).

To further characterize sleep, we examined arousal threshold, which is normally elevated during the sleep-like state at night. Arousal threshold was measured by responsiveness to auditory stimuli (Zhadanova et al., 2001). Nine different intensities were delivered between ZT17 and ZT23, and a sudden change in locomotion was taken as an indicator of responsiveness. A larva was scored as a responder if it moved more than 7 pixels after the stimulus and the average percentage of responders was calculated for each stimulus intensity per group for each experimental run (N = 5 experimental runs). Overall, no difference was seen between ΔCLK larvae and their control siblings (Fig. 7H; Table S3).

Finally, we asked whether there was a difference in the movement of ΔCLK fish compared to control fish in the chamber used for the light dark assay during the subjective day, as may occur if there were an effect on sleep. As seen in Fig. S4, the amount of movement was not different (Cohen’s d = 0.59 [95% CI -0.294, 1.469]; p = 0.182 by Student’s t-test). Together, these results suggest that expressing truncated clocks in the habenula does not affect sleep.

4. Discussion

All aspects of life, from arousal state (Scammell et al., 2017) to the ability to handle stress (Daut and Fonken, 2019) and respond to rewards (Antle and Silver, 2015; McClung et al., 2005), vary with time of day. This reflects the tight control exerted by the circadian clock on the physiology and behaviour of animals. Clock genes, which drive the circadian clock, are expressed broadly in the brain (Moore and Whitmore, 2014; Shieh, 2003; Sun et al., 1997). In this paper, we investigated the hypothesis that the habenula clock influences the circadian variation in response to a stressor. We have found that habenula clock in zebrafish is transcriptionally complex, consisting of multiple paralogs. Given the potential redundancy provided by this, we used expression of a truncated clocka gene (Dekens and Whitmore, 2008) to manipulate the habenula clock. This strategy is based on the discovery in mice that a truncated clocka gene disrupts the circadian clock (Vitaterna et al., 1994), whereas a null mutant does not (DeBruyne et al., 2006). Expression of this antimorphic allele in the zebrafish pineal was previously shown to cause a disruption in the expression of clock-regulated genes and in locomotion and sleep (Livne et al., 2016), indicating that it is an effective technique. Expression in the habenula appears to affect the molecular clock, as indicated by the change in per3 gene expression. We did not observe a complete flattening of per3 expression across the circadian cycle, however, indicating that inhibition is partial. While consistent with previous findings (Dekens and Whitmore, 2008), this level of inhibition appears sufficient to affect habenula function, as indicated by change in day-night variation in neuropeptides that are under the control of the habenula.

As noted in the methods section, ΔCLK fish were obtained by incrossing to maximize expression in the habenula. The number of non-expressing fish were low. It was noticed that there was a group of fish with a low number of fluorescent cells, and these were also used in the control group. The cut-off is arbitrary but appears functionally significant, however, as seen in the light/dark assay. It is possible that non-expressing cells produce ΔCLK, but the HCR analysis indicates that there is a difference between expressing and non-expressing cells. We have used siblings as controls rather than a different set of wildtype fish, to rule out background differences, which are known to influence behaviour in the zebrafish (Audira et al., 2020; van den Bos et al., 2017;
Fig. 7. Sleep is unaltered in Tg(gng8:GAL4, UAS:EGFP-Δclocka) larvae

(A, B) Distances moved (cm/10 min) by an individual fish from (A) Control group and (B) ΔCLK group, plotted against time (hr) over the time-course of the whole recording. CT = circadian time. (C, D) Mean distances moved (cm/10 min) of ΔCLK (blue) and control (orange) groups, plotted against time (hr) during the first 14 h of recording, including 4 h of light-phase and 10 h in the dark during the night (i.e. Zeitgeber Time ZT 10–14 and ZT 14–24) and (D) during the subsequent 24 h of recording under DD (i.e. Circadian Time CT 0–24). 95% CIs are indicated by the shaded areas; horizontal brackets indicate 5 timeframes analysed in panel E. Black and white bars represent periods of dark and light, respectively. (E) The Cumming estimation plot shows the mean differences in 5 timeframes of the recording: ZT10–14 (1; light-phase), ZT14–24 (2; first night), CT0–7 (3: early subjective day), CT7–14 (4: late subjective day) and CT14–24 (5: subjective night). The upper axis shows the raw data, with each dot representing the overall distance moved (cm) by a single larva; gapped vertical lines to the right of each group indicate mean ± SD. Each mean difference is plotted on the lower axes as a bootstrap sampling distribution. Mean differences are depicted as black dots and 95% CIs are indicated by the end of vertical error bars.

(F–H) Analysis of sleep during the subjective night. ΔCLK and control fish have similar average sleep bout (F), sleep bout frequency (G) and total sleep (H). (I) Analysis of sensory responsiveness of the Tg(gng8:GAL4, UAS:EGFP-Δclocka) larvae (7 dpf) during the delivery of auditory stimuli of 9 different intensities. The Cumming estimation plot shows the mean differences for 9 comparisons, corresponding to 9 stimulus intensities (0–100%). The raw data is plotted on the upper axis, each dot representing the percentage of responders per single experimental run. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
To assess the role of the habenula clock, we used the alarm response in zebrafish as a behaviour paradigm. We observed that the freezing behaviour evoked by the alarm pheromone is lower at night compared to the day, and this difference is reduced by expression of the truncated clocka gene specifically in the habenula. We also observed that dark avoidance induced by the alarm substance is reduced by manipulation of the habenula clock. In contrast, the darting and immediate downward movement caused by alarm substance were not affected. No change in circadian behaviours that are unlinked to stressors, such as locomotion or arousal, was observed in fish with ΔCLK expression in the habenula. Together, these observations suggest that the behavioural consequence of disruption of the habenula clock is restricted primarily to anxiety-like behaviour, in contrast to the broader phenotype seen with pineal-specific disruption of the clock (Livne et al., 2019).

One interpretation of these observations is provided by the framework of predictive coding, which has emerged in recent years as a highly effective paradigm for understanding how the brain functions (Clark, 2013; Friston, 2018). A key feature of predictive coding is the updating of expectations based on experience, and this involves the release of neuromodulators that indicate the sign and magnitude of error, as well as precision of prediction. The habenula is one regulator of these modulators, and the pattern of evoked activity and effects of manipulation are consistent with a role in predictive coding underlying motivated behaviour and response to stressors. Recent work has identified a number of mechanisms, such as regulation of membrane potential by astroglial cells (Cui et al., 2018), that influence habenula function by altering the level of spontaneous activity. Spontaneous activity determines the output of a network (Arieli et al., 1996) and is an important element of predictive coding (Hartmann et al., 2015). Here, we propose that the molecular clock, which influences spontaneous activity (Harvey et al., 2020), affects habenula function by altering expectations linked to stressors.

Given the broad expression of clock genes such as per3 and cry1a, it is possible that intrinsic clocks in other components of the network activated by the alarm pheromone e.g. the olfactory bulb, posterior tuberculum or raphe (Jesuthasan et al., 2020), also contribute to circadian variation in the alarm response. Our imaging did not cover this, but work from the Mourrain lab (Leung et al., 2019) indicates that there is circadian variation in neural activity in other regions of the brain. The E-box based clock reporter (Weger et al., 2013) also shows extensive activity. Indeed, there does not appear to be a brain region with no day/night rhythmicity. Nevertheless, the results here establish that disruption of the habenula clock is sufficient to influence the anxiety-like aspect of the response.

Clock gene expression has been reported in the habenula of other vertebrates, including mice (Salaberry et al., 2019). A broad expression of a functional clock in the mammalian habenula is consistent with electrical recordings obtained from slices, which show a change in firing rate and resting membrane potential across the circadian cycle in both medial and lateral habenula (Sakhi et al., 2014a, 2014b; Zhao and Rusak, 2005). The ability of an intrinsic clock to influence habenula-dependent expectations may thus be conserved across vertebrates. This represents a distinct mechanism from the role of clocks in other regions in the regulation of stress (Koch et al., 2017). One implication of the findings here is that identifying clock-regulated genes that regulate spontaneous activity in the habenula may be relevant in the treatment of stress-related disorders.

CRediT authorship contribution statement

Adriana Basnakova: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. Giuseppe D’Agostino: Software, Validation, Formal analysis, Writing – review & editing, Visualization. Sarah R. Langley: Supervision, Writing – review & editing, Funding acquisition. Suresh Jesuthasan: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The link to the code is provided in the methods section.

Acknowledgements

We thank Yoav Gothilf for providing the pT2-UAS:EGFP-2A-ΔCLK-SxMYC construct and Marnie Halpern for providing the TgBAC(gng8:GAL4) line. We also thank Hugh Piggins, David Lyons, Caroline Wee and Ajay Mathuru for comments and discussion, and Erik Meijering for FeatureJ. This work was funded by grants from the Singapore Ministry of Education under its Academic Research Fund Tier 2 (MOE2017-T2-058) and the National Research Foundation ( NRF2017-NRF-ISF002-2676 ) to SJ and an ARAP fellowship from A*Star to AB. SRL was supported by the Lee Kong Chian School of Medicine and Nanyang Technological University Singapore Nanyang Assistant Professor Start-Up Grant, while GDA was supported by the Lee Kong Chian School of Medicine Dean’s Postdoctoral Fellowship. The funding agencies played no role in the study design or decision to publish.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnstr.2021.100403.

References

Agetsuma, M., Aizawa, H., Aoki, T., Nakayama, R., Takahoko, M., Goto, M., Sassa, T., Amo, R., Shiraki, T., Kawakami, K., Honoya, T., Higashijima, S., Okamoto, H., 2010. The habenula is crucial for experience-dependent modification of fear responses in zebrafish. Nat. Neurosci. 13, 1354–1356. https://doi.org/10.1038/nn.2654.
Aibar, Sara, Gonzalez-Blas, Carmen B., Moerman, T., Huyub-Thu, V.A., Imrichova, H., Hulselmans, G., Rambow, Florian, Aerts, Jan, van den Oord, Joost, Atak, Zeynep Kalender, Wouters, Jasper, Aerts, Stein, 2017. SCENIC: single-cell regulatory network inference and clustering. Nat. Methods 14 (11), 1083–1086. https://doi.org/10.1038/nmeth.4463.
Amezquita, R.A., Lun, A.T.L., Becht, E., Carey, V.J., Carpp, L.N., Geistlinger, L., Marini, F., Rue-Albrecht, K., Risso, D., Sonesson, C., Waldron, L., Pages, H., Smith, M. L., Huber, W., Morgan, M., Gottardo, R., Hicks, S.C., 2020. Orchestrating single-cell analysis with Bioconductor. Nat. Methods 17, 137–145. https://doi.org/10.1038/s41592-019-0654-x.
Amo, R., Aizawa, H., Takahoko, M., Kobayashi, M., Takahashi, R., Aoki, T., Okamoto, H., 2010. Identification of the zebrafish ventral habenula as a homolog of the mammalian lateral habenula. J. Neurosci. 30, 1566–1574. https://doi.org/10.1523/jneurosci.3690-09.2010.
Amo, R., Fredes, F., Kinoshita, M., Aoki, R., Aizawa, H., Agetsuma, M., Aoki, T., Shiraki, T., Sakhi, M., Matsuda, M., Yamasaki, M., Takahoko, M., Tsuboi, T., Higashijima, S., Miyasaka, N., Koide, T., Yabuki, Y., Yoshihara, F., Fukui, T., Okamoto, H., 2014. The habenulo-raphe serotonergic circuit encodes an aversive expectation value essential for adaptive active avoidance of danger. Neuron 84, 1034–1048. https://doi.org/10.1016/j.neuron.2014.10.035.
Andalman, A.S., Burns, V.M., Lovett-Barron, M., Broxton, M., Poole, B., Yang, S.J., Groenick, L., Lerner, T.N., Chen, R., Benster, T., Mourrain, P., Levoy, M., Rajan, K., Deisseroth, K., 2019. Neuronal dynamics regulating brain and behavioral state transitions. Cell 177, 970–985. https://doi.org/10.1016/j.cell.2019.02.037 e20.
Pandey, S., Shekhar, K., Regar, A., Schier, A.F., 2018. Comprehensive identification and spatial mapping of habenular neuronal types using single-cell RNA-seq. Curr. Biol.: CB 28, 1052–1065. https://doi.org/10.1016/j.cub.2018.02.040.e7.

Pennartz, C.M.A., Jeu, M.T.G. de, Bos, N.P.A., Schaap, J., Geurtsen, A.M.S., 2002. Diurnal modulation of pacemaker potentials and calcium in the mammalian circadian clock. Nature 416, 286–290. https://doi.org/10.1038/nature728.

Pezzulo, G., Zorzi, M., Corbetta, M., 2021. The secret life of predictive brains: what’s spontaneous activity for? Trends Cognit. Sci. 25, 730–743. https://doi.org/10.1016/j.tics.2021.05.007.

Ren, J., Qin, C., Hu, F., Tan, J., Qiu, L., Zhao, S., Feng, G., Luo, M., 2011. Habenula “cholinergic” neurons co-release glutamate and acetylcholine and activate postsynaptic neurons via distinct transmission modes. Neuron 69, 445–452. https://doi.org/10.1016/j.neuron.2010.12.038.

Sakhi, K., Bell, M.D.C., Benz, S., Hoefer, N., Schmidt, M., Buhl, M., Piggins, H.D., 2014a. Daily variation in the electrophysiological activity of mouse medial habenula neurons. J. Physiol. 592, 587–603. https://doi.org/10.1113/jphysiol.2013.263319.

Sakhi, K., Wegner, S., Bell, M.D.C., Howarth, M., Delagrange, P., Brown, T., Melancon, G., Piggins, H.D., 2014b. Intrinsic and extrinsic cues regulate the daily profile of mouse lateral habenula neuronal activity. J. Physiol. 592, 5025–5045. https://doi.org/10.1113/jphysiol.2014.280065.

Salaberry, N.L., Hamm, E., Felder-Schmittbuhl, M.-P., Mendoza, J., 2019. A suprachiasmatic-independent circadian clock(s) in the habenula is affected by Per gene mutations and housing light conditions in mice. Brain Struct. Funct. 224, 19–31. https://doi.org/10.1007/s00429-018-1756-4.

Satar, N.M.A., Ogawa, S., Parhar, I.S., 2020. Kisspeptin-1 regulates forebrain dopaminergic neurons in the zebrafish. Sci. Rep.-UK 10, 19961. https://doi.org/10.1038/s41598-020-75777-0.

Shieh, K.-R., 2003. Distribution of the rhythm-related genes rPERIOD1, rPERIOD2, and rCLOCK, in the rat brain. Neuroscience 118, 831–843. https://doi.org/10.1016/s0092-8674(00)80004-4.

Smith, R., Badcock, P., Friston, K.J., 2021. Recent advances in the application of predictive coding and active inference models within clinical neuroscience. Psychiatr. Clin. Neurosci. 75, 3–13. https://doi.org/10.1111/pcn.13128.

Speidel, N., Gerlai, R., 2008. Alarm substance induced behavioral responses in zebrafish (Danio rerio). Behav. Brain Res. 188, 168–177. https://doi.org/10.1016/j.bbr.2007.10.031.

Steinbergen, P.J., Richardson, M.K., Champagne, D.L., 2011. Patterns of avoidance behaviours in the light/dark preference test in young juvenile zebrafish: a pharmacological study. Behav. Brain Res. 222, 15–25. https://doi.org/10.1016/j.bbr.2011.03.025.

Stringer, C., Pachitariu, M., 2019. Computational processing of neural recordings from calcium imaging data. Curr. Opin. Neurbiol. 55, 22–31. https://doi.org/10.1016/j.conb.2018.11.005.

Sun, Z.S., Albrecht, U., Zhaocheng, O., Bailey, J., Eichele, G., Lee, C.C., 1997. B Grim. a putative mammalian ortholog of the Drosophila period gene. Cell 90, 1003–1011. https://doi.org/10.1016/s0092-8674(00)80366-9.

Takahashi, J.S., 2017. Transcriptional architecture of the mammalian circadian clock. Nat. Rev. Genet. 18, 164–179. https://doi.org/10.1038/nrg.2016.150.

Tama, T.K., Young, L.C., Whitmore, D., 2007. Light signaling to the zebrafish circadian clock by Cryptochrome 1a. Proc. Natl. Acad. Sci. Unit. States Am. 104, 14712–14717. https://doi.org/10.1073/pnas.0709581104.

Thines, G., Vandenbussche, E., 1966. The effects of alarm substance on the schooling behaviour of r asbora heteromorpha Duncker in day and night conditions. Anim. Behav. 14, 296–302. https://doi.org/10.1016/s0003-4726(56)80866-6.

Trivedi, V., Choi, H.M.T., Fraser, S.E., Pierce, N.A., 2018. Multidimensional quantitative analysis of mRNA expression within intact vertebrate embryos. Development 145, dev158869. https://doi.org/10.1242/dev.158869.

van den Bos, R., Mes, W., Galligani, P., Heil, A., Zetholz, J., Flik, G., Gorissen, M., 2017. Further characterisation of differences between TL and AB zebrafish (Danio rerio): gene expression, physiology and behaviour at day 5 of the larval stage. PloS One 12, e0175420. https://doi.org/10.1371/journal.pone.0175420.

Vitaterna, M., King, D., Chang, A., Kornhauser, J., Lowrey, P., McDonald, J., Dove, W., Pinto, L., Turek, F., Takahashi, J., 1994. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science 264, 719–725. https://doi.org/10.1126/science.8171325.

Wang, H., Yang, Z., Li, X., Huang, D., Yu, S., He, J., Li, Y., Yan, J., 2020. Single-cell in vivo imaging of cellular circadian oscillators in zebrafish. PLoS Biol. 18, e3000435. https://doi.org/10.1371/journal.pbio.3000435.

Weger, M., Wegler, B.D., Dietel, N., Rastegar, S., Hirota, T., Kay, S.A., Strahle, U., Dickmeis, T., 2013. Real-time in vivo monitoring of circadian E-box enhancer activity: a robust and sensitive zebrafish reporter line for developmental, chemical and neurological biology of the circadian clock. Dev. Biol. 380, 259–273. https://doi.org/10.1016/j.ydbio.2013.04.035.

Wilkinson, S., Dodgson, G., Meezes, K., 2017. Predictive processing and the varieties of psychological trauma. Front. Psychol. 8, 1840. https://doi.org/10.3389/fpsyg.2017.01840.

Woods, G.L., Schopfik, D., Shi, V.J., Zimmerman, S., Coleman, H.A., Greenwood, J., Soucy, E.B., Schier, A.F., 2014. Neuropeptidergic signaling partitions arousal behaviors in zebrafish. J. Neurosci. 34, 3142–3160. https://doi.org/10.1523/jn.0175420.13.2014.

Yang, Y., Cui, Y., Sang, K., Dong, Y., Ni, Z., Ma, S., Hu, H., 2018. Ketamine blocks bursting in the lateral habenula to rapidly relieve depression. Nature 554, 317–322. https://doi.org/10.1038/s41586-018-02559.

Zappia, L., Oshlack, A., 2018. Clustering trees: a visualisation for evaluating clusterings at multiple resolutions. Gigascience 7, giy083. https://doi.org/10.1093/gigascience/giy083.

Zhang, H., Rusak, B., 2005. Circadian firing-rate rhythms and light responses of rat habenular nucleus neurons in vivo and in vitro, 132, pp. 519–527. https://doi.org/10.1016/j.neuroscience.2005.01.012.

Zhdanova, I.V., Wang, S.Y., Leclair, O.U., Danilova, N.P., 2001. Melatonin promotes sleep–like state in zebrafish. Brain Res. 903, 263–268. https://doi.org/10.1016/s0006-3499(01)02444-1.