# The habenula clock in zebrafish underlies circadian variation in response to a stressor

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# Abstract

The lateral habenula, a regulator of neuromodulator release, contains a semi-autonomous molecular clock whose significance is unknown. Here, using zebrafish, we investigate the contribution of this clock to change in internal state that is triggered by an aversive stimulus. We focused on the alarm pheromone, *Schreckstoff*, whose effects vary in a circadian manner. Disruption of habenula clock function, by selective expression of a truncated clock gene (ΔCLK), altered the effects of *Schreckstoff* such that larval responses in the day resembled those at night. Acute responses, including immediate change in swim speed, were unaffected, whereas extended behaviours such as freezing in a novel environment and dark avoidance, were reduced. Circadian behaviours that are under the control of the pineal clock, such as locomotion and sleep, were unchanged. These findings suggest that the habenula clock can act independently to influence the internal state induced by an aversive stimulus.


# Introduction

Sensory stimuli evoke a variety of responses in an animal. A stimulus with high salience, for example, will elicit changes in movement and in internal state. The changes in state, which can include increase in arousal and vigilance, are mediated by the release of neuromodulators such as serotonin (Chaouloff et al., 1999) and dopamine (Abercrombie et al., 1989; Holly and Miczek, 2016). One regulator of these neuromodulators in vertebrates, especially in response to aversive stimuli, is the habenula (Namboodiri et al., 2016). The habenula, which is located in the epithalamus, consists of two domains, medial and lateral, with the latter controlling dopamine and serotonin release from the ventral tegmental area (VTA) and raphe, respectively. Exposure to a stressor increases the level of activity in lateral habenula neurons (Andalman et al., 2019), and abnormal firing in the lateral habenula is associated with maladaptive behaviours (Li et al., 2013; Yang et al., 2018). Thus, a knowledge of factors that influence activity in the lateral habenula is important in understanding what shapes the response to stressors.

One such a factor appears to be an intrinsic molecular clock. Components of the clock machinery (Takahashi, 2017), such as *per2* in mouse (Guilding et al., 2010) and rat (Zhao et al., 2015), *per1* and *bmal*1 in zebrafish (Weger et al., 2013), and *per1b* in goldfish (Sánchez-Bretaño et al., 2015), are expressed in a circadian manner in the lateral habenula. Although the

habenula receives input from the master pacemaker in the suprachiasmatic nucleus (SCN) (Buijs, 1978; Sakhi et al., 2014), the habenula clock persists even when the SCN is lesioned (Salaberry et al., 2019). In general, the molecular clock drives daily variations in excitability, enabling neurons to generate rhythmic electrical output (Belle, 2015; Cao and Nitabach, 2008; Pennartz et al., 2002). In agreement with this, spontaneous firing in the habenula of rat (Zhao and Rusak, 2005) and mouse (Guilding et al., 2010; Sakhi et al., 2014) varies across a circadian cycle. It has been shown that the effect of a stressor on lateral habenula electrical activity is largest during the active period, when spontaneous activity is lowest (Chastrette et al., 1991). An implication of this is that the habenula clock influences the response to stimuli that are processed by the habenula. There is, however, no experimental evidence for this.

We hypothesize that the habenula clock acts in an autonomous manner to influence response to an aversive stimulus. It is known that the response to stressors varies with time of day (Koch et al., 2017), but the underlying mechanism is unclear. One strategy that can be used to investigate the function of the molecular clock in a specific cell is to express a truncated CLOCK protein (ΔCLK) (Dekens and Whitmore, 2008). The molecular clock consists of a transcriptional-translational feedback loop (Takahashi, 2017). A heterodimer of CLOCK and BMAL enters the nucleus, where it drives transcription of *period* (*per*) and *cryptochrome* (*cry*) genes, which then inhibit the heterodimer. The truncated CLOCK protein binds BMAL, but the resulting heterodimer is unable to activate transcription of target genes. This strategy has been successfully used in the pineal of zebrafish, where expression of truncated CLOCK prevented circadian variation in clock-controlled genes in the pineal and led to a disruption of circadian rhythmicity in locomotion, sleep and vertical position (Ben-Moshe Livne et al., 2016). Here, we use the GAL4/UAS system to drive expression of ΔCLK specifically in the habenula of zebrafish. Our results demonstrate that the intrinsic habenula clock has an essential role in regulating the behavioural response to an aversive stimulus.

# Results

## The zebrafish habenula displays circadian variation in activity

The expression of clock genes in the mouse habenula is correlated with circadian changes in neural activity (Guilding et al., 2010; Sakhi et al., 2014). To assess whether there is a circadian change in neural activity in the zebrafish habenula, we used immunohistochemical detection

77 of phosphorylated extracellular signal-regulated kinase (pERK), which has the advantage of
78 reporting neural activity in freely swimming animals (Randlett et al., 2015); this method has
79 been used to examine circadian activity in the mouse SCN (Hughes et al., 2004). In 8 day old
80 zebrafish, pERK label was evident in both nuclei and the cytoplasm of scattered neurons.
81 Generally, pERK label was more prominent in the lateral (or ventral) habenula compared to
82 the medial (or dorsal) habenula (compare cells indicated with white arrowheads in Figure 1A
83 and B). Label was also seen in neuropils (Figure 1A). To determine if there is any consistent
84 difference in activity between day and night, registered and scaled image data was first
85 normalized by calculating the pERK/total-ERK (tERK) ratio. The normalized image data was
86 then compared between 'Day' and 'Night' groups, which consisted of > 100 zebrafish larvae
87 fixed at Zeitgeber time ZT3 (3 hours after lights on; Figure 1D, G) and ZT15 (Figure 1C, F)
88 respectively. A pixel-wise 'Night'-over-'Day' comparison, implementing a false discovery rate
89 threshold of 0.1%, (Figure 1E, H) indicates that levels of pERK were higher at night relative
90 to the day, especially in the lateral habenula. The day-night difference was also seen in the
91 lateral habenula of fish held in constant darkness (Figure 2), although to a lesser extent than
92 fish that had undergone a normal light-dark cycle. This suggests that, while light has a role,
93 neural activity in the lateral habenula is influenced by a circadian clock.

## 94 Disruption of the habenula clock with ΔCLK

95 To selectively express ΔCLK in the habenula, we used the GAL4/UAS system with
96 *TgBAC(gng8:GAL4)* (Hong et al., 2013) as the driver. In addition to ΔCLK, the effector
97 construct also included enhanced green fluorescent protein (EGFP), which was separated from
98 ΔCLK by a 2A peptide linker, thus enabling the expression of two separate proteins. The
99 presence of EGFP in the habenula neurons of *Tg(gng8:GAL4, UAS:EGFP-ΔCLK)* fish (Figure
100 3A, B) indicates that the construct is expressed appropriately and enables convenient
101 identification of ΔCLK+ individuals (Ben-Moshe Livne et al., 2016). Labelling with an
102 antibody against the Myc tag, which is fused in frame at the C-terminus of ΔCLK, indicates
103 that the truncated CLOCK protein is translated (Fig. 3C). To assess whether ΔCLK expression
104 had an effect on physiological function of the habenula, brains were isolated from adult
105 zebrafish expressing EGFP-ΔCLK, and from their non-expressing siblings, at two different
106 time points, ZT3 and ZT15. The supernatant from extracts were analysed by liquid
107 chromatography and mass spectrometry (LC/MS) (Chatterjee and Gerlai, 2009). While non-
108 expressing fish had a clear difference in the day- and night-time levels of dopamine (DA), there

was almost no difference in fish expressing ΔCLK protein (Figure 4). The difference in day-night serotonin (5-HT) levels was also reduced in the ΔCLK+ fish, although to a lesser extent. These results indicate that expression of ΔCLK in the habenula is effective in disrupting the intrinsic molecular clock.

## Habenula expression of ΔCLK affects the alarm response

We hypothesized that the behavioural responses to an aversive stimulus would be affected by the expression of ΔCLK. One aversive stimulus for fish is the alarm pheromone *Schreckstoff* (Pfeiffer, 1962). This substance elicits sustained activity in the lateral habenula (Jesuthasan et al., 2020). The alarm response shows circadian variation in harlequin fish (Thines and Vandenbussche, 1966) and bleak (Jachner, 1996), with a strong response seen primarily in day, i.e. the active period. To test whether this is also the case in zebrafish, we exposed larval fish to the alarm substance either at 11 am or 11 pm. Larval zebrafish respond to the alarm substance by increased immobility (Jesuthasan et al., 2020). To sensitively detect this, a vibration was presented five minutes before and after delivery of the alarm substance. Control zebrafish showed a reduction in startle after delivery of the alarm substance during the day, but not at night (Figure 5), consistent with freezing during the day but not at night. *Tg(gng8:GAL4, UAS:EGFP-ΔCLK)* fish did not show a reduction in startle at day or at night. These data suggest that the alarm response is circadian in zebrafish and is influenced by the habenula clock.

The habenula is involved in the regulation of neuromodulators, which would be expected to alter behaviour that reflect change in state, rather than immediate changes in locomotion in response to a stimulus. In adult zebrafish, *Schreckstoff* causes an acute change in swimming speed and tank position (Jesuthasan and Mathuru, 2008; Speedie and Gerlai, 2008). Additionally, fish transiently exposed to *Schreckstoff* display persistent stress, as indicated by abnormal scototaxis and elevated levels of serotonin (Maximino et al., 2014), as well as increased alertness (Pfeiffer and Riegelbauer, 1978). 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. Adult *Tg(gng8:GAL4, UAS:EGFP-ΔCLK)* zebrafish, like their non-expressing siblings, showed an increase in darting and moved to the base of the tank, with a number of fish then freezing (Figure 6A-E). To monitor post-exposure effects, individual fish were exposed to the alarm substance in a beaker, then transferred to a novel tank. ΔCLK fish, in contrast to their non-

141 expressing siblings, did not freeze, but behaved similarly to fish that had been exposed to fish
142 water (Figure 6F, G). We also monitored the post-exposure behaviour of larval zebrafish, using
143 a light-dark choice assay (Steenbergen et al., 2011). In comparison to their non-expressing
144 siblings, larval *Tg(gng8:GAL4, UAS:EGFP-ΔCLK*) fish displayed less dark avoidance after
145 transient exposure to *Schreckstoff*, as indicated by the amount of time spent in the dark side of
146 the tank (Figure 6H, I). Together, these observations indicate that disruption of the habenula
147 clock leaves acute responses unaffected but reduces the persistent stress response that is evoked
148 by transient exposure to the alarm substance.

## 149 Expression of ΔCLK in the habenula does not affect pineal-regulated circadian 150 behaviour

151 We hypothesized that while the circadian clock in the habenula affects the response to an
152 aversive stimulus, circadian behaviours that are under the control of the pineal clock (Ben-
153 Moshe Livne et al., 2016) should be unaffected. To test this, we analysed the rhythmic
154 locomotor activity (Prober et al., 2006) of ΔCLK+ larvae and their control (CTRL) siblings.
155 All larvae were entrained to the standard light-dark (LD) cycle and their locomotion was
156 monitored for a few hours during the light phase, followed by the onset of darkness at ZT14
157 for activity tracking during the night. Subsequently, the fish were monitored for one day under
158 constant darkness (DD), in order to eliminate the masking effects of light and dark on clock-
159 regulated behaviour (Ben-Moshe Livne et al., 2016). As seen in Figure 7, ΔCLK+ larvae
160 showed a similar pattern of locomotion as their non-expressing siblings, becoming mobile
161 during the light phase and during the subjective day, while exhibiting quiescent periods of
162 inactivity during the 1st and 2nd night of the recording.
163
164 In addition to locomotion, we also examined arousal threshold, which is normally elevated
165 during the sleep-like state at night. Arousal threshold was measured by responsiveness to
166 auditory stimuli (Zhdanova et al., 2001). Nine different intensities were delivered between
167 ZT17 and ZT23, and a sudden change in locomotion was taken as an indicator of
168 responsiveness (Figure 7F, G). A larva was scored as a responder if it moved more than 7 pixels
169 after the stimulus and the average percentage of responders was calculated for each stimulus
170 intensity per group for each experimental run (N = 5 experimental runs). Overall, no difference
171 was seen between ΔCLK+ larvae and their control siblings (Figure 7I). Together, these
172 experiments indicate that disruption of the habenula clock has relatively specific effects.

173

174 # Discussion

175 All aspects of life, from arousal state (Scammell et al., 2017) to the ability to handle stress
176 (Daut and Fonken, 2019) and respond to rewards (Antle and Silver, 2016; McClung et al.,
177 2005), vary with time of day. This reflects the tight control exerted by the circadian clock on
178 the physiology and behaviour of animals. Clock genes, which drive the circadian clock, are
179 expressed broadly in the brain (Moore and Whitmore, 2014; Shearman et al., 1997; Shieh,
180 2003; Sun et al., 1997). In this paper, we investigated the hypothesis that the habenula clock
181 influences the circadian variation in response triggered by a stressor. Such stimuli are known
182 to trigger the release of neuromodulators, which cause an extended change in internal state and
183 thus in behaviour (Anderson and Adolphs, 2014), and the habenula is a part of the circuit
184 mediating stimulus-mediated neuromodulator release. To test this hypothesis, we expressed a
185 dominant negative mutant of the *clock* gene (Dekens and Whitmore, 2008) specifically in the
186 zebrafish habenula. We examined how this affected the alarm response of zebrafish, which we
187 show here to be a circadian behaviour, as has been reported in other fish (Jachner, 1996; Thines
188 and Vandenbussche, 1966).
189
190 Expression of ΔCLK protein in the habenula led to a reduced alarm response, such that the
191 day-night difference was no longer present. Disruption of the clock inhibited freezing and dark
192 avoidance induced by the alarm substance. The darting and immediate downward movement
193 caused by alarm substance were not affected, consistent with the hypothesis that the habenula
194 primarily affects the change of internal state that follows exposure to the alarm substance. No
195 change in circadian behaviours that are unlinked to stressors, such as locomotion or arousal,
196 was observed in fish with ΔCLK expression in the habenula. Together, these observations
197 indicate that the behavioural consequence of disruption of the habenula clock is restricted
198 primarily to stress responses, in contrast to the broader phenotype seen with pineal-specific
199 disruption of the clock (Ben-Moshe Livne et al., 2016).
200
201 One possible mechanism for the reduced stress response in fish expressing ΔCLK is that the
202 net change in stimulus-evoked activity is lower. As shown in this paper, activity in the zebrafish
203 lateral habenula is higher at night. Expression of ΔCLK blocks transcription of *per* (Dekens
204 and Whitmore, 2008), which is predicted to place habenula neurons in the 'night state'

throughout the circadian cycle. Consistent with this, the day-time levels of dopamine and serotonin in ΔCLK fish were similar to night-time levels. A higher level of activity means that an incoming stimulus will cause a relatively small change in output. In contrast, the same stimulus will have a stronger effect in a fish with low spontaneous activity in the habenula. This interpretation is in line with the finding of Chastrette and colleagues (Chastrette et al., 1991) that the effect of a stressor on the habenula of rats is higher during the active period, when spontaneous activity is low. The observation that the alarm response is reduced at night is consistent with this.

Mechanisms that control spontaneous activity in the lateral habenula are one area of growing interest in mental health, given that increased activity here is linked to abnormal stress responses (Andalman et al., 2019; Li et al., 2013; Yang et al., 2018). The findings here suggest that a potential way forward is to manipulate the clock specifically in the habenula, given the relatively specific effects seen. It may also be useful to screen for clock-regulated genes. This could be done by single-cell RNAseq of habenula neurons at different times of the day, for example, which may lead to the identification of ion channels or other molecules that regulate intrinsic activity and excitability of habenula neurons. This knowledge may contribute to an improvement in the treatment of stress disorders.

# Materials and Methods

## Transgenic lines

TgBAC(gng8:GAL4)$^{c426}$ (Hong et al., 2013) was used to drive expression in the habenula. Tg(UAS:EGFP-ΔCLK) was generated by injection of pT2-UAS:EGFP-2A-ΔCLK-5xMYC using Tol2 mediated transgenesis. All experiments were carried out under guidelines approved by the IACUC of Biopolis (181408) and NTU (A19014).

## pERK analysis

***Immunohistochemistry***

Fish of *nacre* background were randomly split into 'Day' and 'Night' groups. Fish were raised at a density of ~0.125 fish/mL and maintained at 28°C under 14-10 h LD cycle, henceforth referred to as standard conditions. For the comparison under DD, tanks with the larvae were transferred into a dark box at ZT14 the day before fixation.

235

236 Larvae at the age of 8 dpf were fixed at Zeitgeber time ZT3 and ZT15 or circadian time CT3
237 and CT15, based on findings that the peak and trough of *per3* expression occurs at ZT3 and
238 ZT15, respectively (Moore and Whitmore, 2014). Immediate and consistent fixation was
239 achieved by pouring the water containing the larvae through a fine sieve that was immediately
240 placed in ice-cold 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Fixations
241 at ZT15, CT3 and CT15 were performed under dim light. Incubation in the fixative was carried
242 out at 4ºC for ~22h. Fixed larvae were then stored in 100% methanol at -20℃ for several days.
243 Larvae were rehydrated by successive 5-minute washes in 70%, 50%, 25% and 0% methanol
244 in PBS, then placed in PBST [1 % Bovine serum albumin (Sigma) + 1 % dimethyl sulfoxide
245 (DMSO) + 0.1% Triton-X 100 in PBS] for 1 h at room temperature. They were then incubated
246 in primary antibodies ((rabbit anti-pERK (Cell Signalling, #4370) and mouse anti-tERK (Cell
247 Signalling, #4696), both diluted at 1:500) at 4°C for 3-4 days. Samples were washed five times
248 in PBST and then incubated in secondary antibodies (Alexa Fluor 647 goat anti-rabbit
249 (ThermoFisher) and Alexa Fluor 488 goat anti-mouse (ThermoFisher), both diluted at 1:500)
250 for 2 h at room temperature. Samples in which the primary antibody was omitted were run in
251 parallel to test the specificity of the secondary antibody binding. Sample size was based on
252 previous experiments (Randlett et al., 2015).

253 ***Confocal imaging***

254 Larvae were mounted in 2% low melting agarose (LMA) in PBS and imaged using a Zeiss
255 LSM800 upright confocal microscope equipped with 40X 1.0 NA water dipping objective. The
256 acquisition settings were kept consistent in all experiments. Images were collected at 512x512
257 resolution. The habenula was imaged with parts of the forebrain (pallium and subpallium) to
258 improve image registration. The number of frames was set to cover the whole depth of habenula
259 and varied across samples, which was later corrected during the registration.

260 ***Image registration and data analysis***

261 In order to efficiently compare pERK signals between 'Day' and 'Night' groups, all confocal
262 stacks were first aligned so that each voxel covered an approximately equivalent physical
263 location in each sample. This was achieved by non-rigid registration of the images to a single
264 reference image using the homogenous anti-tERK staining. The registration was carried out
265 with the Computational Morphometry Toolkit (CMTK, http://www.nitrc.org/projects/cmtk/)
266 and the command string (-awr 010203 -T 4 -X 52 -C 8 -G 80 -R 3 -A '--accuracy 0.4' -W '--
267 accuracy 1.6'). Registered stacks were scaled down to 150/150/30 (x/y/z) resolution and the

268 image noise was reduced with 2D Gaussian filter (sigma = 2) using the
269 'PrepareStacksForMAPMapping.ijm' macro in FIJI software
270 (http://engertlab.fas.harvard.edu/Z-Brain/ (Randlett et al., 2015)). Image data was then
271 normalized by calculating pERK/tERK ratio and a pairwise comparison between 'Day' and
272 'Night' groups was carried out at each pixel as follows: first pseudo groups were randomly
273 generated containing equal number of fish from both groups and Mann-Whitney U statistics
274 Z-score was calculated over 500 iterations, producing a distribution of Z-scores. Assuming the
275 normal distribution, a Z-score value corresponding to the false discovery rate of 0.1% was then
276 used as a threshold so that if the Z-score value of the 'Day' vs 'Night' comparison was higher
277 than the threshold it was considered significant. Application of the false discovery rate of 0.1%
278 as a threshold was justified by a control comparison of randomly created mixed groups. The
279 pixels which were significantly different during the final 'Night'-over-'Day' comparison,
280 during which 'Day' group served as a baseline, were then assigned a colour where green and
281 magenta represented significant increase and decrease, respectively, in the 'Night' group.
282 Those pixels were also allocated intensity values within the range of 0:65535 according to the
283 size of delta between the median values of the two groups (i.e. the lowest and highest intensities
284 indicated the smallest and largest deltas within the range of 0:0.5, correspondingly). The pixels
285 which did not exhibit significant difference were not assigned any colour. All data were
286 analysed using the 'MakeTheMAPMap.m' script (http://engertlab.fas.harvard.edu/Z-Brain/,
287 (Randlett et al., 2015)) in Matlab (Mathworks).

## 288 Immunohistochemistry and imaging of EGFP-ΔCLK

289 The *Tg(gng8:GAL4,UAS:EGFP-ΔCLK)* larvae at the age of 7 dpf were fixed in 4% PFA at 4ºC
290 for ~22 h. For immunostaining of extracted brains, skin covering the brain was manually
291 removed and exposed brains were washed 3 times in PBST. Antigen retrieval was performed
292 by incubating the fish in 150 mM Tris-HCl (pH 9.0) for 5 min at room temperature in order to
293 equilibrate, and by subsequent heating of the samples at 70˚C for 15 min. Following several
294 washes and a blocking step, samples were incubated in the rabbit anti-GFP primary antibody
295 (Torrey Pines Biolabs Inc., #TP401), diluted 1:500, and the mouse anti-Myc antibody (9E10;
296 Santa Cruz Biotechnology), diluted 1:100, at 4ºC for a few days. The secondary antibodies
297 used were Alexa Fluor 488 goat anti-rabbit (ThermoFisher and Abcam), diluted 1:1000, and
298 Alexa Fluor 647 goat anti-mouse (ThermoFisher), diluted 1:500. The immunostained brains

299 were mounted in 2% LMA in PBS and imaged using a Zeiss LSM800 confocal microscope
300 equipped with 40X 0.7 NA water immersion objective.

## 301 Screening and selection of *Tg(gng8:GAL4,UAS:EGFP-ΔCLK)* fish

302 All of the *Tg(gng8:GAL4,UAS:EGFP-ΔCLK)* fish used in the following experiments were first
303 screened for GFP fluorescence at 3 dpf using a Olympus MVX10 stereo fluorescence
304 microscope. Since the expression of EGFP-ΔCLK in the habenula was mosaic, only individuals
305 with expression in the majority of habenula cells were selected and used for further experiments
306 as ΔCLK-positive (ΔCLK+) fish. Individuals with zero expression as well as larvae exhibiting
307 a minimal number of randomly labelled cells (less than ~15 cells per habenula) were used as
308 the control (CTRL) siblings (Figure S1). Following the behavioural experiments, larvae were
309 immobilized in tricaine methanesulfonate (MS-222; final concentration of 0.01 mg/ml; Sigma),
310 mounted in 2% LMA in E3 medium and the habenula-specific expression of EGFP was
311 assessed using a Zeiss LSM800 confocal microscope and 40X water dipping objective.

## 312 Quantification of DA and 5-HT levels

313 Three-month old female ΔCLK+ fish and their CTRL siblings were immobilized using iced
314 water and euthanized by decapitation at ZT3 and ZT15. Whole brains, excluding the olfactory
315 bulb, were extracted in the ice-cold Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM
316 $CaCl_2$, 5.0 mM HEPES, pH 7.2), snap-frozen in liquid nitrogen and stored in -80˚C until further
317 analysis. Supernatant was collected as described in (Chatterjee and Gerlai, 2009).
318 Quantification of dopamine (DA) and serotonin (5-HT) was carried out by LC/MS using the
319 following analytical standards: dopamine hydrochloride (D2960000: Sigma-Aldrich),
320 dopamine-1,1,2,2-$d_4$hydrochloride (73483: Sigma-Aldrich) and serotonin (14927: Sigma-
321 Aldrich). Data were analysed and plotted using Estimation Statistics (Ho et al., 2019). Three
322 biological replicates (i.e. measurements of different samples) were performed.

## 323 Behaviour assays

### 324 *Acoustic startle following exposure to Schreckstoff*

325 Thirteen to sixteen day-old ΔCLK larvae and their non-expressing siblings were placed in two
326 transparent plastic tanks with dimensions of 80 x 54 x 33 (L x W x H in mm) and their
327 locomotor activity was recorded from above at 10 fps using a camera (acA2040-90µm USB
328 3.0; Basler). Each of the two tanks was filled with 20 mL of facility water and held 8 fish at

329 most. The water level in each tank was 5 mm in height to minimize vertical movement. The
330 alarm substance was freshly prepared from adult zebrafish (Mathuru et al., 2012) each week,
331 and was tested for effectiveness on adults before use on larvae. An Arduino Grove Vibration
332 Motor was positioned in between the two plastic tanks, on the stage holding the tanks, to
333 provide the stimulus. The vibration was 1 sec in duration and was presented 3 times, at 1 minute
334 intervals, starting five minutes before and after the delivery of the *Schreckstoff*. A startle
335 response was defined as a change in body orientation of more than 90 degrees within 5 sec in
336 response to each vibration tone. The response (yes or no for each fish) was averaged across 3
337 tones for each group to obtain a response percentage before and after the delivery of the
338 *Schreckstoff*. Both the vibration tones and the delivery of *Schreckstoff* were controlled by an
339 Arduino board synched with the video-recording Python codes. Experiments were performed
340 on 3 separate batches of embryos. A different batch of freshly prepared skin extract was used
341 for each experiment; the extract was verified for effectiveness by testing on adult fish. Only
342 extract that evoked freezing was used.
343
344 ***Locomotor activity***
345 The *Tg(gng8:GAL4,UAS:EGFP-ΔCLK)* larvae were raised under standard conditions. Each
346 experimental run involved animals from the same batch, originating from one pair of parents.
347 The larvae were screened and selected as described in the previous section. At the age of 6 dpf
348 the larvae were placed individually in a 24-well plate filled with 1.5 ml of tank water per well.
349 The plate was then transferred into an incubator equipped with an IR camera (acA2040-90µm
350 USB 3.0: Basler), a white LED box and four IR LED bars (LBS2-00-080-3-IR850-24V,
351 850 nm: TMS Lite). The camera and the LEDs were connected to a PC via a microcontroller
352 board (Arduino UNO SMD). At the onset of recording (ZT6 – 9), fish were kept under ambient
353 light, which was switched off at ZT14 (10 PM) and was kept off during the next day for the
354 monitoring of activity under DD. A customized script written in Python 2.7, incorporating
355 functions from the OpenCV library was used to control the Arduino microcontroller and
356 simultaneously video-track fish locomotor activity. The video was captured using 576 x 864-
357 pixel resolution at 10 frames per second and the background subtraction method was
358 implemented to extract the 'x' and 'y' coordinates of each fish online. The position data was
359 then converted to total distance (mm) travelled by each fish per 1 min time. The data
360 conversion, further analysis and plotting were carried out using custom-written Excel macros,
361 Python 2.7 and Estimation Statistics (Ho et al., 2019).

362 ***Arousal threshold***

363 Six days-old ΔCLK+ larvae and their CTRL siblings were randomly placed in a 24-well plate
364 and their locomotor activity was video-tracked as described in the previous section. In order to
365 assess the arousal during sleep, vibration stimuli were applied through a speaker controlled,
366 via an Arduino microcontroller board, by a modified version of the activity-tracking code in
367 Python 2.7. Starting at ZT17, eighteen stimuli of nine different intensities, where 50% of
368 computer audio output represented the highest intensity, were delivered for 200 ms (starting
369 from 0%) in 12-16% increments every minute, first in a descending and then ascending order.
370 The time interval between stimuli was determined based on previous findings that suggest a
371 30-second inter-stimulus gap is sufficient to avoid behavioural habituation (Burgess and
372 Granato, 2007). The protocol was repeated at the commencement of every hour until ZT22,
373 while the onset by ascending or descending order was altered each time. In total, 12 replicates
374 (i.e. technical replicates) of each stimulus intensity were generated. A fish was scored as
375 responding if it moved more than 7 pixels (~ 4 mm) following the stimulus delivery and the
376 average percentage of responding fish was calculated for each of the stimulus intensities
377 (Woods et al., 2014). The observed values were corrected for variations in the baseline
378 locomotor activity, by first calculating the background probability of locomotion as a
379 percentage of larvae which moved more than 7 pixels 5 s before the application of each
380 stimulus. The average of 108 measures of the background probability of locomotion was then
381 used to calculate the corrected probability of response at each of the stimulus intensities,
382 implementing the following equation: corrected probability of response = observed value x
383 [(observed value - background offset) / (max observed value - background offset)] (Woods et
384 al., 2014).

385 ***Novel tank assay***

386 All experiments were conducted between ZT2 and ZT4 on 3-5 month-old adult zebrafish,
387 following the protocol of (Haghani et al., 2019)). Fish were recorded in pairs in a darkened
388 room with a black background, where tanks were illuminated from the top with a natural light
389 LED bar. Novel tanks were made of glass measuring 20 cm (L) by 5 cm (W) by 12 cm (H)
390 with opaque sides (to prevent fish from viewing each other), placed side by side. Fresh water
391 from the facility was added to tanks up to the 10 mL mark to make a total volume of 1 L. The
392 setup was placed within a curtained area to obscure the experimenter.
393

394 Prior to exposure to the novel tank, fish were handled carefully to ensure that handling stress
395 was minimised. Fish were gently netted in pairs from their home tanks in the facility to smaller
396 crossing tanks, where they were brought into the room with the behavioural setup. Then, fish
397 were netted into 100 mL beakers with ~30 mL of fish facility water and quickly poured into
398 the novel tank. The recording was started within 30 s of fish being in the recording tanks. For
399 exposure to *Schreckstoff,* fish were placed in 100 mL beakers containing 200 µL of heated skin
400 extract (see (Chia et al., 2019) for extraction method) in 50 mL fish facility water for 2 minutes.
401 Fish were netted into another beaker containing fresh facility water before being poured into
402 the novel tank. Videos were acquired at 10 frames per second for 600 s (10 mins) using a Basler
403 Ace camera (acA1300–200 µm; 1,280 × 1,024). The position (x-y coordinates) of the fish were
404 tracked, and speed was binned by mm/s into a probability density curve.
405
406 For acute exposure to *Schreckstoff*, fish were first habituated in the glass tanks for 5-7 mins.
407 Then, fish were recorded in the tanks for 5 mins before 1 mL of 1:4 dilution of heated skin
408 extract was pipetted into the tanks. The recording continued for 5 mins after *Schreckstoff*
409 delivery for the alarm response to be observed. Two fish were tested at one time, each in a
410 separate tank, with one tank containing ΔCLK fish and the other a non-expressing sibling. The
411 tank used for a particular genotype was switched at every trial. Characteristic alarm behaviours
412 were quantified, such as time spent in the bottom third of the tank, number of darting episodes
413 (fish speed >5 SD above average swim speed for the first 5 mins), and number of freezing
414 episodes (movement of <5.5 mm per second). Tracking was automated to prevent observer
415 bias. Sample size was based on previous experiments (Chia et al., 2019).

416 ***Light/dark choice assay***

417 All experiments were performed between ZT2 and ZT9 in a behavioural arena within blackout
418 curtains as described previously (Cheng et al., 2016). Four transparent plastic tanks
419 (dimensions: 43 mm W × 60 mm L × 30 mm H) filled with 30 mL fresh tank water served as
420 assay chambers. Opaque cardboard sheets were placed between the 4 tanks. Tanks were placed
421 on an Apple iPad screen and videos (9 fps) were recorded on a USB3.0 Basler camera (Model#
422 acA2040-90umNIR 1440 × 1080 pixels) with a long-pass filter (MIDOPT, LP830 830nm)
423 placed above. Two IR light bars (850 nm peak from TMS-lite) positioned next to the 4 tanks
424 served as an IR light source. The light/dark compartments were created by white and black
425 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 minutes. 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 minutes. 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.

# Acknowledgements

We thank Yoav Gothilf for providing the pT2-UAS:EGFP-2A-ΔCLK-5xMYC construct and Marnie Halpern for providing the *TgBAC(gng8:GAL4)*$^{c426}$ line. We also thank Hugh Piggins, David Lyons, Caroline Wee and Ajay Mathuru for comments and discussion. 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 by an ARAP fellowship from A*Star to AB.

## Author contributions

AB, RKC and JCSM performed experiments and analysed data. SJ and AB designed the study and wrote the manuscript.

# Figures

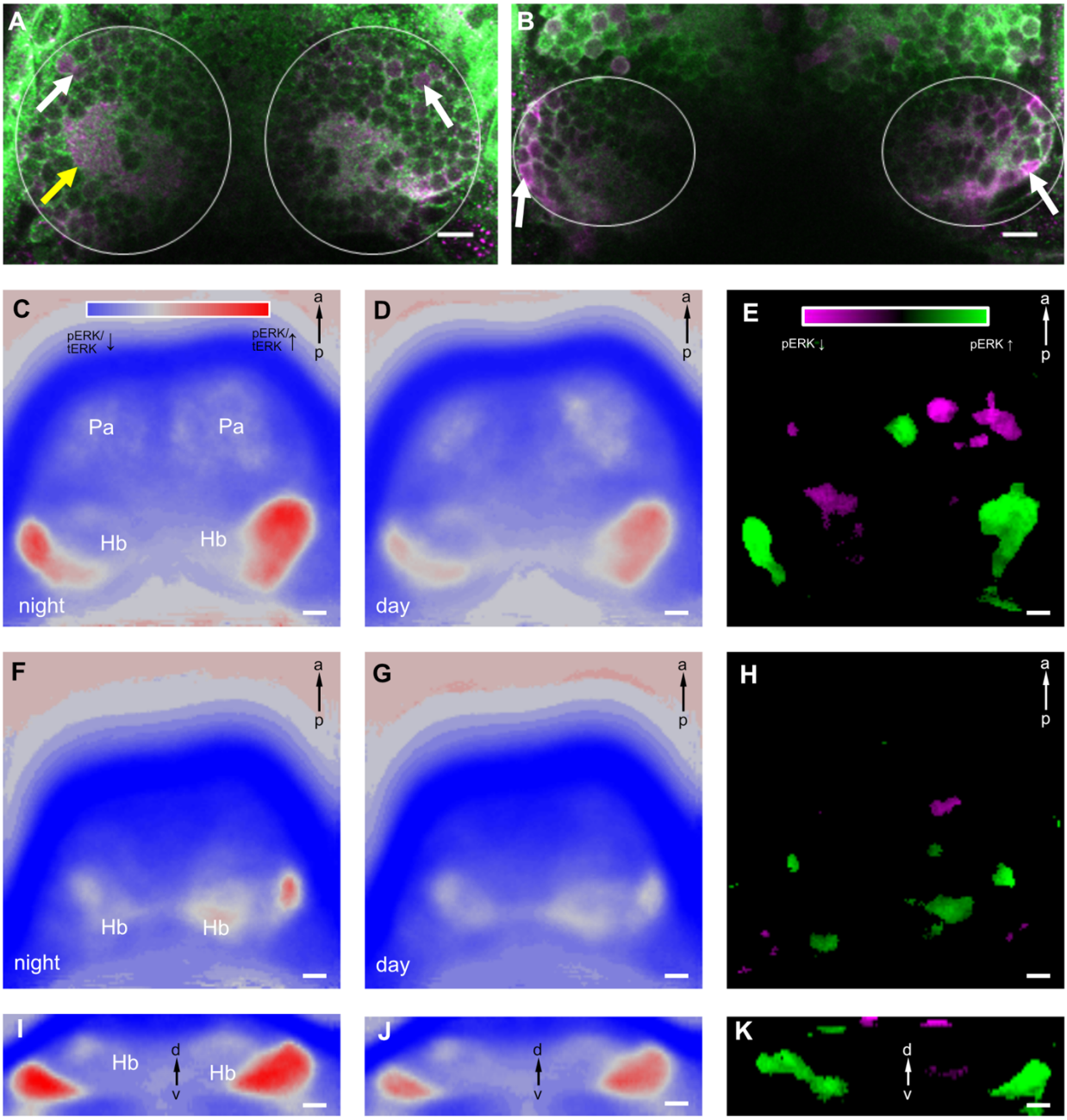



**Figure 1. Day-night difference in ERK phosphorylation in the habenula.** (**A, B**) Immunohistochemical detection of phosphorylated ERK (pERK; magenta) and total ERK (tERK, green) at two different focal planes of the habenula, dorsal (A) and ventral (B), of an 8-day old zebrafish. The white arrows indicate labelled cell bodies, while the yellow arrow indicates the neuropil. (**C-H**) Day-Night comparison of normalized pERK levels in the habenula. Raw images were registered, downsampled and smoothed and pERK/tERK ratio was calculated at each pixel. Median pERK/tERK levels in the habenula were calculated based on N = 102 larvae fixed during the night (C, F) and N = 130 larvae fixed during the day (D, G).

Two different planes, ventral (C, D) and dorsal (F, G) are shown here. The difference in the ventral (E) and dorsal (H) planes, based on 'Night-over-Day' comparison, with green indicating higher levels of pERK at night, while magenta indicates lower levels (see wedge in panel E). (**I-K**) Reconstructed transverse section through the habenula of the stacks of median activity during the night (I), day (J) and difference (K). Strong activity is detected in the lateral region only. All images except I-K are dorsal view. Scale bar = 10 µm. a: anterior; p: posterior; d: dorsal; v: ventral; Pa: pallium; Hb: habenula.

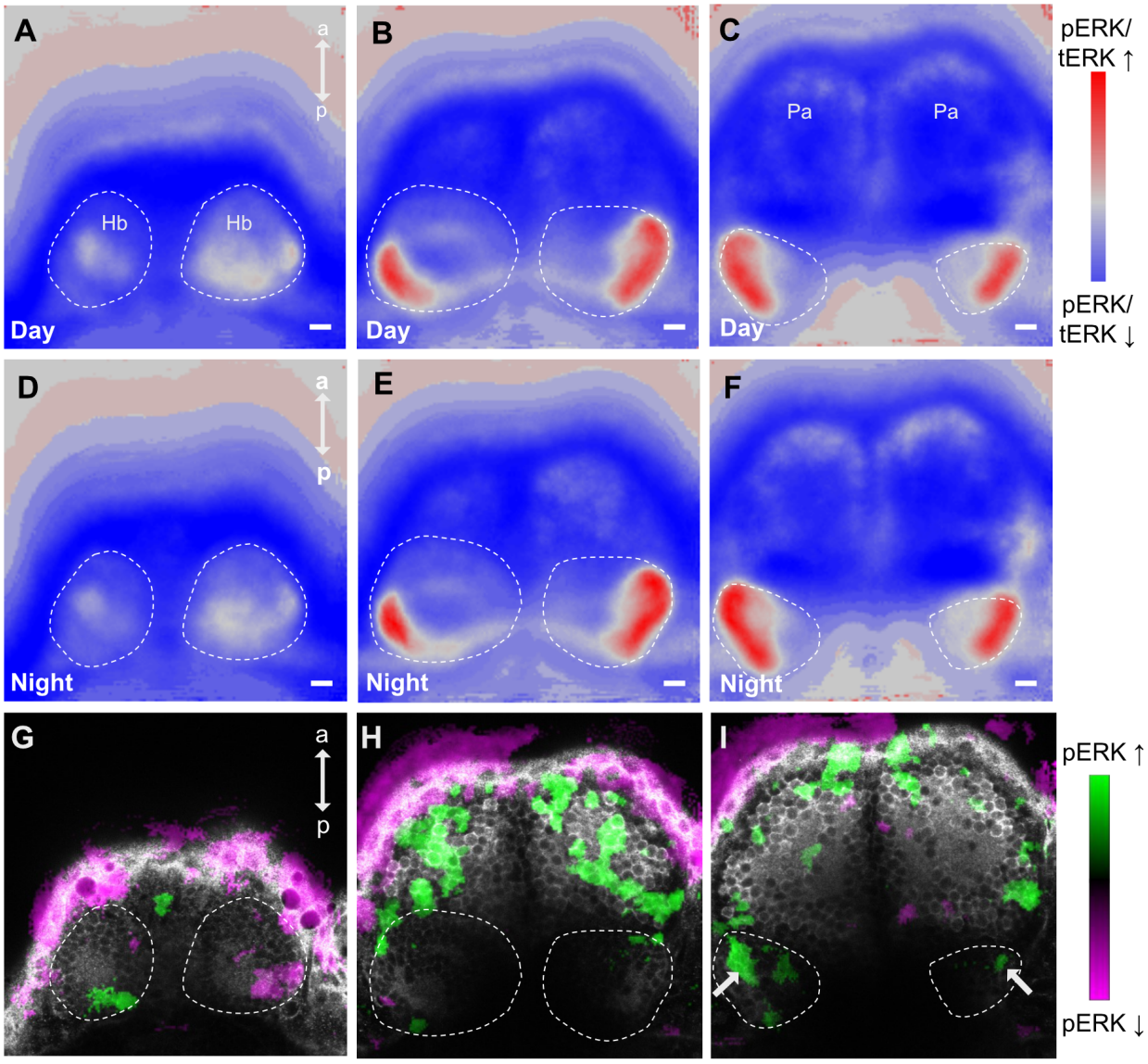
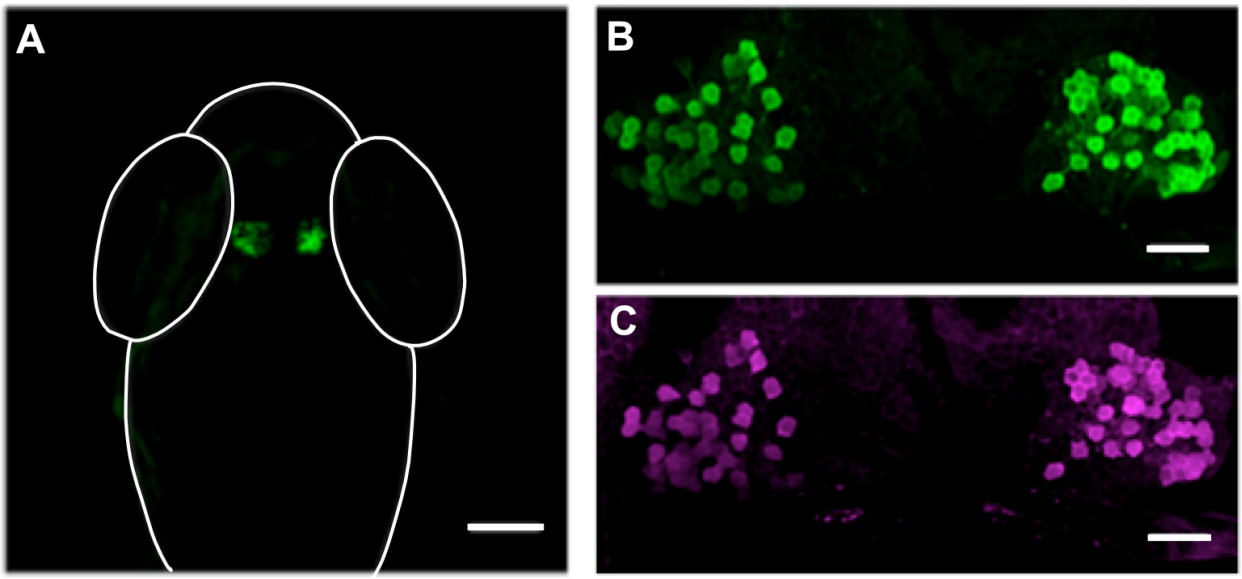



**Figure 2. Circadian variation in habenula activity under constant darkness.** (**A-F**) Median pERK/tERK label at three different focal planes, in larvae fixed in the day (**A-C**; N = 104) or at night (**D-F**; N = 102). A and D are dorsal, B and E are intermediate, whereas C and F are deep. (**G-I**). The difference in pERK levels at Night versus Day, at the 3 different focal planes.

472 The arrows in I indicate increased pERK in the lateral habenula. Bar = 10 µm. Contrast settings
473 on panels A-F are identical.
474
475




476
477 **Figure 3. The *gng8:*GAL*4* driver enables expression of a C-terminus truncated *clock* gene**
478 **in the habenula of zebrafish.** (**A**) Specific expression of EGFP in the habenula of a
479 Tg(*gng8:GAL4, UAS:EGFP-ΔCLK*) fish. (**B**) At higher magnification, eGFP is visible in the
480 cytoplasm of habenula neurons. (**C**) Detection of Myc, which is fused to the C-terminus of
481 ΔCLK, in the habenula of transgenic fish. Scale bar = 100 µm in panel A, 20 µm in panels B
482 and C.
483
484

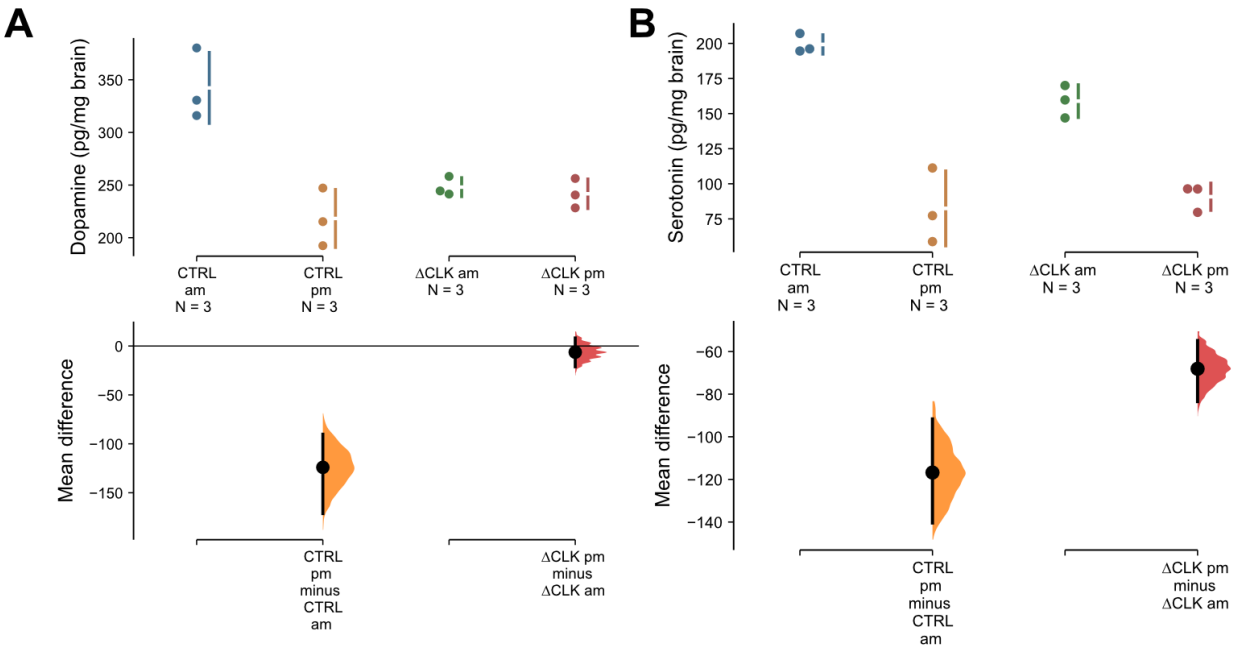



**Figure 4. Effects of ΔCLK expression in the habenula on global neuromodulator levels.** Cumming estimation plots showing the difference in levels of dopamine (A) and serotonin (B) at daytime and night-time in ΔCLK and sibling fish. The raw data is plotted on the upper axis, with each dot representing a value obtained from a single brain. Gapped vertical lines to the right of each group indicate mean ± SD. Mean differences are depicted on the lower axis as black dots, with the vertical bars indicating bootstrap 95% CIs.

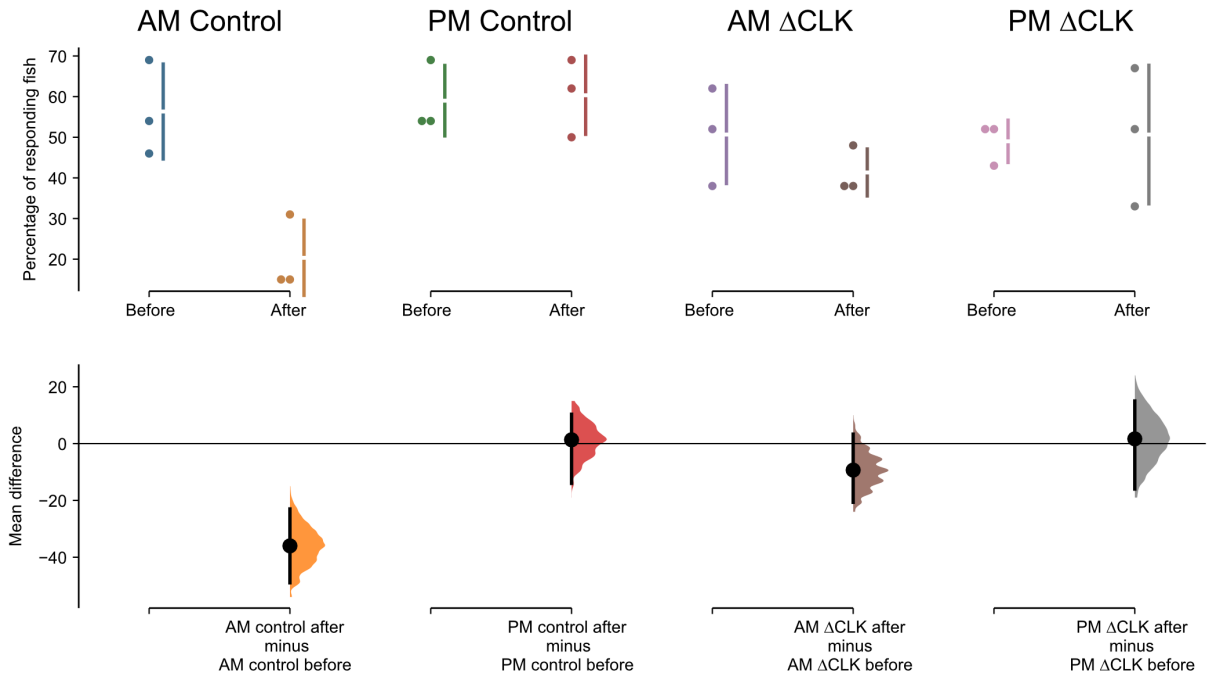



**Figure 5. Day-night variation in the response of zebrafish to *Schreckstoff*.** Percentage of larval fish that show a startle response before and after exposure to the alarm substance, during the day (11 am) or at night (11 pm). Control fish (N = 13), which are non-expressing siblings of *Tg(gng8:GAL4, UAS:ΔCLK)* fish (N = 21), show a reduction in startle after exposure during the day, but not at night. Each circle in the top plot represents the percentage of responding fish in one batch. The bottom half shows a Cummings estimation plot of the difference between the startle after and before exposure to the alarm substance. Mean differences are depicted as dots; 95% confidence intervals are indicated by the ends of the vertical error bars.

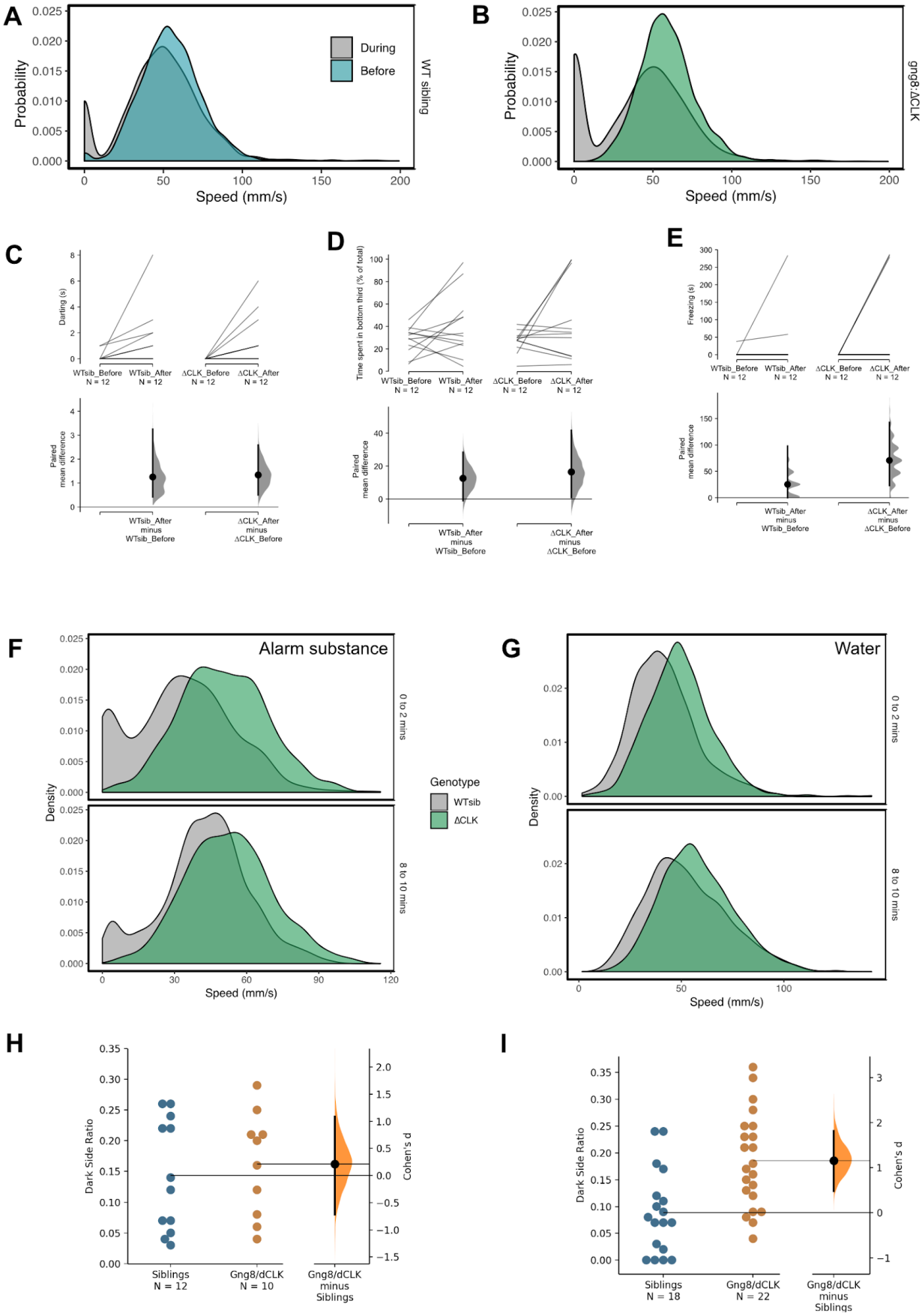



**Figure 6. The effects of *Schreckstoff* on *Tg(gng8:GAL4, UAS:EGFP-ΔCLK)* fish. (A-E)** Acute effects in adult zebrafish. (**A, B**) Speed distribution in fish before and during exposure

to the alarm substance Freezing, as indicated by the peak at 0 mm/s, is seen in both wildtype (A) and ΔCLK fish (B). (**C-E**) Time spent darting (C) or at the bottom third of the tank (D), or freezing (E) before and in the presence of *Schreckstoff*. There is no difference in the behaviour of fish with and without expression of truncated clocka. (**F, G**). Post-exposure behaviour in adult fish (N = 8). (**F**) Histogram of speed in a novel tank containing clean water, after exposure to Schreckstoff in a separate container. Fish expressing ΔCLK in the habenula do not freeze (green shading), in contrast to their non-expressing siblings (gray shading). (**G**). Behaviour of non-exposed fish in the novel tank. No freezing was observed. (**H, I**) Post-exposure behaviour in 2 week-old zebrafish. (**H**) Percentage of time in the dark area of a tank, after 10 minutes of exposure to embryo water. (**I**) Percentage of time spent in the dark side after exposure to the alarm substance. *Tg(gng8:GAL4, UAS:EGFP-ΔCLK)* fish spend more time in the dark side, with a Cohen's d of 1.2.

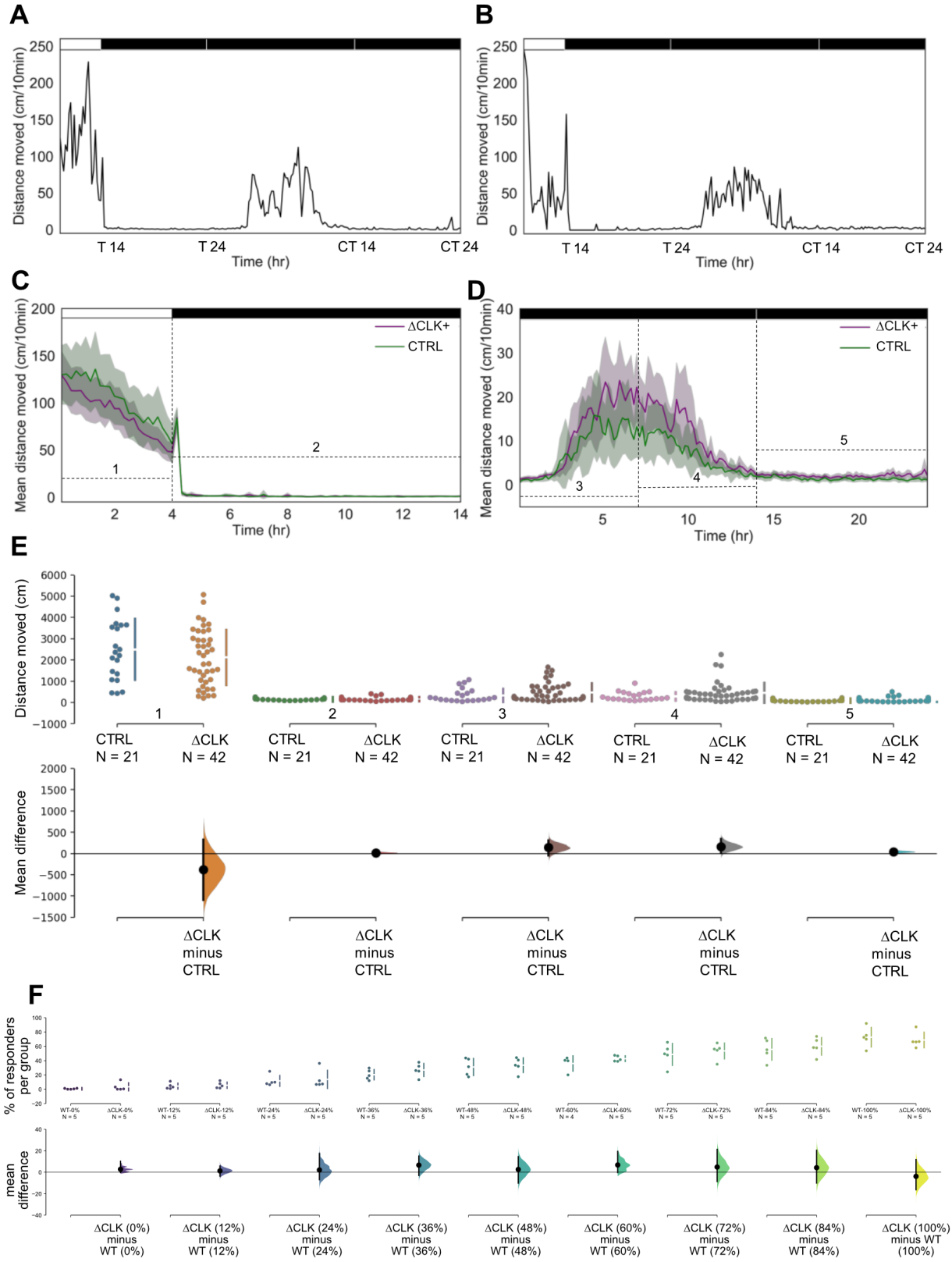
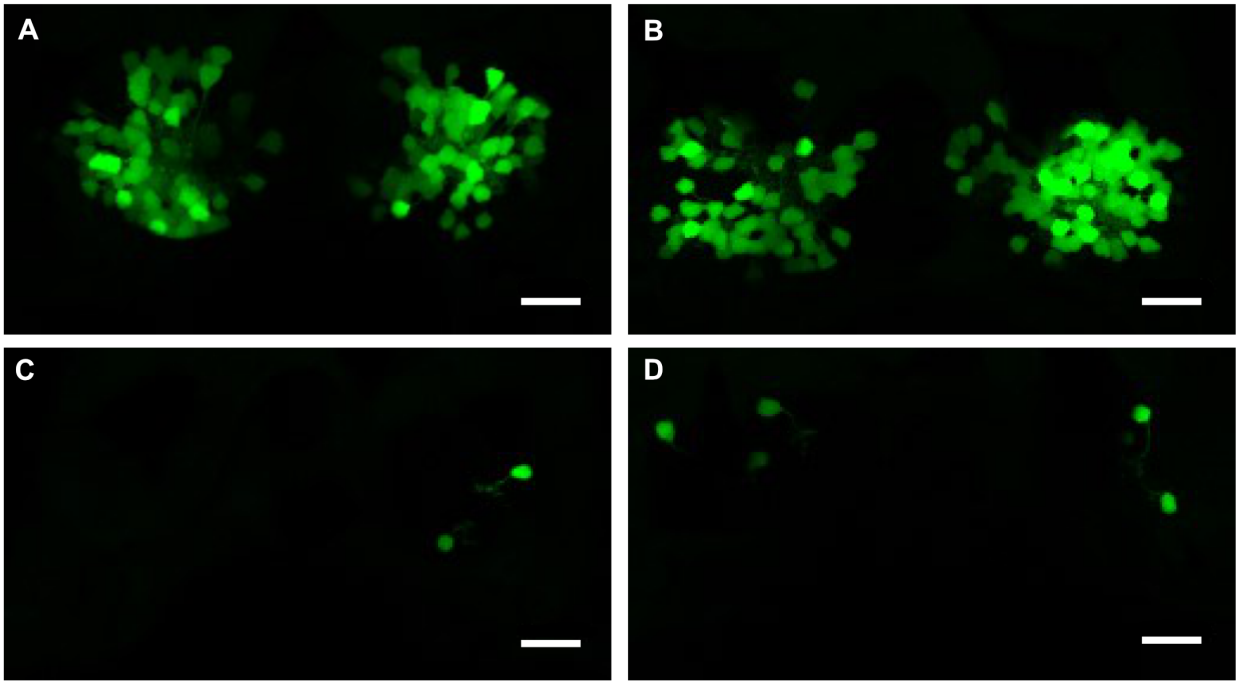



**Figure 7. Circadian behaviour is unaltered in *Tg(gng8:GAL4, UAS:EGFP-ΔCLK)* larvae**

520 (**A, B**) Distances moved (cm/10min) by individual larvae from (A) CTRL group and (B)
521 ΔCLK+ group are plotted against time (hr) over the time-course of the whole recording. CT =
522 circadian time. (**C, D**) Mean distances moved (cm/10min) are plotted against time (hr) (C)
523 during the first 14 hours of recording, including 4 hours of light-phase and 10 hours in dark
524 during the night and (D) during the subsequent 24 hours of recording under DD. CTRL (green)
525 groups Tg(gng8:GAL4;UAS:EGFP-ΔCLK) 95% CIs are indicated by the shaded areas;
526 interrupted lines indicate 5 analyzed timeframes. Black and white bars represent periods of
527 dark and light, correspondingly. (**E**) The Cumming estimation plot shows the mean differences
528 for 5 comparisons, corresponding to 5 timeframes of recording. These are 1 = light-phase; 2 =
529 1st night; 3 = early subjective day; 4 = late subjective day; 5 = subjective night. The raw data
530 is plotted on the upper axis, each dot representing the overall distance moved (cm) by a single
531 larva; gapped vertical lines to the right of each group indicate mean ± SD. Each mean difference
532 is plotted on the lower axes as a bootstrap sampling distribution. Mean differences are depicted
533 as black dots and 95% CIs are indicated by the end of vertical error bars. (**F**) Analysis of
534 sensory responsiveness of the *Tg(gng8:gal4,UAS:EGFP-ΔCLK)* larvae (7 dpf) during the
535 delivery of auditory stimuli of 9 different intensities. The Cumming estimation plot shows the
536 mean differences for 9 comparisons, corresponding to 9 stimulus intensities (0-100%). The raw
537 data is plotted on the upper axis, each dot representing the percentage of responders per single
538 experimental run. (G)
539




540
541 Figure S1. Examples of *Tg(gng8:GAL4, UAS:EGFP-ΔCLK)* larval zebrafish with broad (A, B)

542 and sparse (C, D) expression of EGFP in the habenula. All images are dorsal view. Scale bar
543 = 10 μm.

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